Evaluating the Role of Early HIV-Specific T and B Cell Phenotypes and Interaction in Determining Downstream Antibody Function in HIV Infection

Thesis

EVALUATING THE ROLE OF EARLY HIV-SPECIFIC T AND B CELL PHENOTYPES AND INTERACTIONS IN DETERMINING DOWNSTREAM ANTIBODY FUNCTION IN HIV INFECTION

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Dedication

I dedicate my PhD work to my family: my wife, Beth Sidi Oyaro, my brothers, Brian Mireri and Kevin Nami, my sisters, Rose Kwamboka and Faith Moraa, my late grandmother, Mary Karuru and more so, my parents, Engineer Charles Oyaro and Mrs. Consolata Naitore, whose faith in me, taught me to have faith in myself. You are all very special and may God bless you all.

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ACK	Ammonium-Chloride-Potassium
ADCC	Antibody-dependent cellular cytotoxicity
ADCD	Antibody-dependent complement deposition
ADCP	Antibody-dependent cellular phagocytosis
ADCVI	Antibody-dependent cell-mediated virus inhibition
ADNK	Antibody-dependent natural killer
AIDS	Acquired immunodeficiency Syndrome
AIM	Activation Induced Marker
AM	Atypical memory
AM	Activated memory
AU	Arbitrary Units
BAFF	B cell Activating Factor
BCL6	B cell lymphoma 6 protein
BCR	B cell Receptor
Blimp-1	B cell lymphocyte-induced maturation protein-1
bnAbs	Broadly neutralising functions
Bregs	Regulatory B cells
BSA	Bovine Serum Albumin
CCR	C-C chemokine receptor
CD	Cluster of Differentiation
CD4bs	CD4 binding site
CDRs	Complementarity-determining regions
CMV	Cytomegalovirus

COVID-19	Coronavirus disease 2019
CTLA-4	Cluster of differentiation 154
DMSO	Di-Methyl Sulfoxide
DNA	Deoxyribonucleic Acid
EBV	Epstein–Barr virus
EDI	Estimated date of infection
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ENV	Envelope
ERC	Ethical Review Committee
Fab	**F**ragment **a**ntigen **b**inding
FACS	Fluorescence-activated cell sorting
Fc	**F**ragment **c**rystallizable
FCS	Foetal Calf Serum
FDC	Follicular dendritic cells
FSW	Female sex workers
GALT	Gut Associated Lymphoid Tissue
GC	Germinal centre
HCV	Hepatitis C virus
HEPES	Hydroxyethyl piperazineethanesulfonic acid
IAVI	International AIDS Vaccine Initiative
ICOS	Inducible T cell co-stimulator
ID50	50% inhibitory dose
IDU	Intravenous drug users

IFN- α	Interferon alfa
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFNs	type I interferons
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
IRF4	Interferon regulatory factor 4 (IRF4)
KAVI	Kenya AIDS Vaccine Initiative
KAVI-KNH	Kenya AIDS Vaccine Initiative-Kenyatta National Hospital
KEMRI-CGMRC	Kenya Medical Research Institute - Centre for Geographic Medicine Research-Coast
LEF	Lymphoid enhancer-binding factor
LMCV	Lymphocytic Choriomeningitis virus
MFI	Median fluorescence intensities
MHC	Major histocompatibility complex
ml	Millilitre
MLV	Murine retrovirus
MPER	Membrane-proximal external region
MSMs	Men who have sex with men
NK	Natural killer
nm	Nanometre
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBST	PBS containing 0.1% Tween 20

PeP	Post-exposure prophylaxis
pg	Picogram
pHSCs	Pluripotent hematopoietic stem cells
PI	Post-HIV infection
pMHC	Peptide-MHC complex
PMTCT	Prevention of mother to child transmission
PreP	Pre-exposure prophylaxis
RM	Resting memory
RNA	Ribonucleic acid
sd	Standard deviation
SEB	Staphylococcal enterotoxin B
SHM	Somatic hypermutation
SIV	Simian immunodeficiency virus
STAT	Signal transducer and activator of transcription
TCF	Transcriptional factor
TCR	T cell receptor
Tfh	T follicular helper
TGF	Transforming growth factor
TLM	Tissue-like memory
TLRs	Toll-like receptors
ug	microgram
UNAIDS	United Nations Programme
WHO	World Health Organization

Abstract

There is limited knowledge on how to induce potent antiviral antibodies in HIV vaccine strategies. Studies of HIV infected individuals who develop potent anti-HIV antibodies could inform rational vaccine immunogen design approaches. Phenotypic characteristics of T and B cells, as well as interactions between these cells early in infection may affect the quality of antiviral antibodies. This study investigated the impact of the general and HIV-specific T and B cell phenotypes and interactions in early HIV infection on subsequent anti-HIV antibody quality.

Using a cohort of 53 antiretroviral-naive HIV-infected adults that were longitudinally followed for approximately 48 months, the general and HIV-specific T and B cell subsets, as well as plasma CXCL13 and B cell-activating factor levels, as proxy for germinal centre activities, were determined in early infection and their association with subsequent development of antibody levels and their neutralising and Fc-mediated functions assessed.

Atypical B cells were elevated in HIV, (20% [IQR, 12.15-29.55] versus 5.4% [IQR, 3.45-6.67] in HIV-naive donors, $p<0.0001$), whereas CD4+ T cells were depleted (41.6% [IQR, 32.2-53.1] versus 50.4% [IQR, 48.1-55.65], $p=0.004$). HIV-specific B and T-follicular helper (Tfh) cells were detected in infected individuals, 0.58% (IQR, 0.41%-0.9%) and 0.54% (IQR, 0.3-0.83), respectively. Antibody neutralization breadth was detected in 28.3% of the participants, while Fc-mediated functions were common, with 15.1% of the individuals showing quality Fc-polyfunctionality over time.

The Tfh subset, ICOS+ PD-1+ CXCR5+ CD4+, was associated with subsequent higher IgG titres (rho=0.3135, $p=0.025$), while HIV-specific Tfh cells predicted interferon-gamma release by natural killer cells (rho=0.3314, $p=0.018$). Activated B cells were positively associated with higher IgG_1 levels (rho=0.4285, $p=0.0014$). Similarly, HIV-specific B cells and CXCL13 levels

were associated with higher IgG_3, (rho=0.3, p=0.029), and IgG levels (rho=0.37, p=0.006), respectively.

The data suggest that early T and B cell features can be predictive of downstream antibody functions and levels. Immunological signatures identified early in HIV infection could be useful in shepherding desired antibody responses.

Chapter 1 Introduction

1.1: The biology of HIV

Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV), a lentivirus, that has its origins from chimpanzees (*Pan troglodytes*) following transmission of the chimpanzee simian immunodeficiency virus (SIVcpz) to humans [1]. Divergent simian precursors led to the emergence of two related viruses, HIV-1 and HIV-2 [2, 3]. HIV-1 is the most pathogenic and is responsible for the worldwide pandemic. It comprises of four distinct lineages: groups M, N, O, and P. Group M is the most prevalent in the world, while group O, N and P are less prevalent, but capable of causing AIDS [4-6]. Group M has nine genetically distinct clades that may recombine to form hybrid forms of the virus. The clades include: A, B, C, D, F, G, H, J and K. Similarly, HIV-2 comprises of eight groups: A, B, C, D, E, F, G and H.

HIV-1 is a ribonucleic acid (RNA) retrovirus that has 9 genes encoding 15 proteins [7]. The *gag, pol* and *env* genes are the dominant genes that encode for structural proteins and enzymes. Gag proteins, the matrix, capsid, nucleocapsid and p6 give rise to the core of the virion, whereas the surface Envelope (Env) protein gp120 and transmembrane Env protein gp41 give rise to the outer membrane. The reverse transcriptase, integrase and protease are enzymes that play a critical role in HIV replication. The other genes *vif*, *vpr*, *vpu* and *nef* encode for accessory proteins while Tat and Rev are regulatory proteins. Specifically, Vpu indirectly assists in virion release and evasion of host immune responses while Tat and Rev provide vital gene regulatory functions. Vif, Vpr, and Nef aid in immune evasion and promote virus replication [7]. For instance, Nef enhances infectivity of CD4+ T cells by activating them [8] and disrupts phagocytosis of HIV infected cells by reducing major histocompatibility complex (MHC) class I expression [9]. The disruption of the MHC class I trafficking by Nef leads to enhanced viral persistence since immune recognition by cytotoxic T lymphocytes is abrogated.

Viral entry begins with contact between gp120 and a CD4+ molecule on the target cell. This contact leads to conformational changes in gp120, thereby exposing the CCR5+ or CXCR4+ co-receptor binding site, which leads to the fusion of the virion's Env with the host cell membrane. Once the viral core is delivered into the cytoplasm, viral transcripts are expressed, and Tat-dependent reverse transcription occurs. Viral deoxyribonucleic acid (DNA) in the form of a pre-integration complex is integrated into the host's genome, forming a provirus. In the nucleus, proviral transcription, catalysed by host polymerase II, leads to transcription products which are then transported from the nucleus to the cytoplasm for translation, a step regulated by Rev.

After successful translation in the endoplasmic reticulum, Gag, Gag-Pol and Env polyproteins are localized to the cell membrane. By assembling Gag and Gag-Pol polyproteins, Vif, Vpr, Nef and the genomic RNA to form the core particle, an immature virion is formed which buds out of the host cell and detaches. The immature virion will be mature and infectious once the proteolytic processing of Gag and Gag-Pol polyproteins is complete [7, 10, 11]. Figure 1.1 gives an overview of the HIV replication cycle.

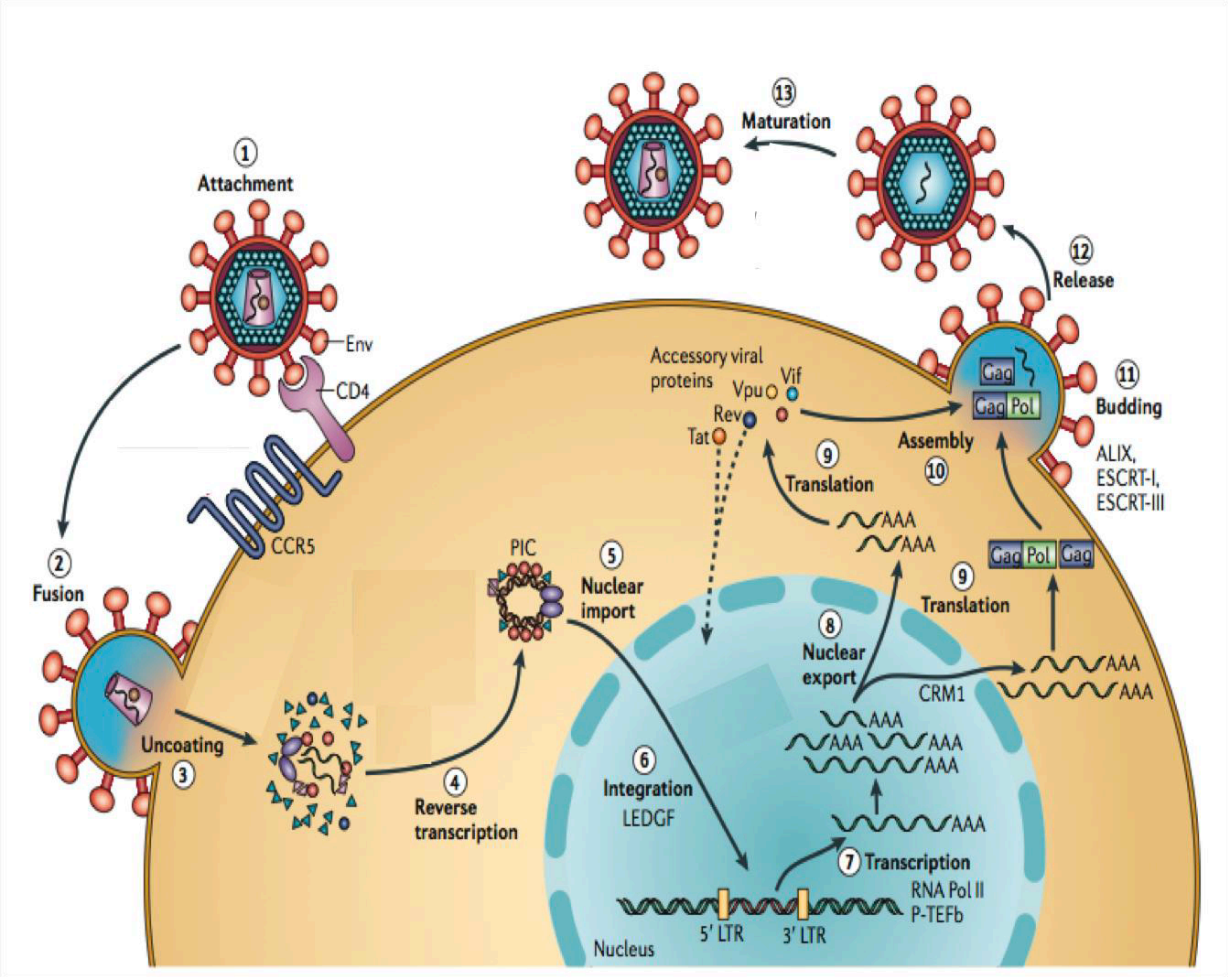


Figure 1:1: The overview of the HIV replication cycle. HIV begins by attaching, which leads to viral–cell membrane fusion and entry of the virus particle into the cell. Core shell uncoating facilitates reverse transcription. After reverse transcription, the pre-integration complex is exported to the nucleus and is integrated, transcripted before viral mRNAs are exported out of the nucleus to the cytoplasm. In the cytoplasm, viral mRNA is translated, and the proteins assembled. The immature virion then buds out of the host cell and matures to become infectious. Adapted from [10].

1.2: Transmission, disease progression and stages of untreated HIV infection

It is still not clear if HIV is transmitted as a cell-bound or free virus [12]. However, in a macaque model study, cell-free simian immunodeficiency virus (SIV) was shown to infect the animals more efficiently than cell-associated infection by both vaginal and intravenous route [13]. This compelling evidence suggested that cell-free HIV is likely to be the preferred mode of HIV transmission, as compared to the cell-associated mode. HIV infection is instigated by

a lone viral variant that is derived from the quasi-species of the transmitting individual [14] and the virus may take up to 10 days before p24 antigen and viral RNA copies are detectable in blood [15]. However, in some instances, such as in cases of men who have sex with men (MSMs), intravenous drug use or even in genital inflammation associated HIV transmission, multiple founder viruses may be transmitted [16-18]. The transmission of a lone viral variant, despite the high diversity of HIV population in the donor, has been termed as the "transmission bottleneck" and the underlying mechanisms for this phenomenon remain incompletely understood. What is known is that multiple exposures are required for effective transmission and that intraepithelial dendritic cells may be the first cells to be infected, and thereby spreading the infection to other immune cells.

Once transmitted, viral replication is enhanced by mechanisms mediated by innate cells [19] mainly through the recruitment of susceptible CD4+ T cells and the production of immunosuppressive cytokines. The presentation of virally infected cells by dendritic cells to activated CD4+ CCR5+ T cells at draining lymph nodes has widely been suggested to push for viral spread in the body [20]. Also, the binding of HIV-specific B cells to HIV through complement receptor CD21 has been shown to spread the virus [21]. These B cells are found both in peripheral blood and lymphoid organs and may then infect susceptible HIV-specific CD4+ T cells.

Sixty per cent of infected CD4+ T cells become activated and die by apoptosis [22]. However, the B cell population with the bound virus is steady, although their function may remain impaired due to the destruction of germinal centres (GCs) in lymphoid organs [23]. As such, immune dysfunction is triggered soon after a successful HIV transmission, setting in the acute phase of HIV infection. Heterosexual sex is the primary route of transmission [24], but

homosexuality practices [25] and intravenous drug use [26] remain key risk factors of HIV transmission.

The acute phase of HIV infection refers to the period immediately after HIV acquisition, when viremia increases exponentially, and widespread destruction of immune cells occurs. The acute period varies from individual to individual, but generally lasts for about 4 weeks and is characterised by very high rates of HIV infectiousness [27]. The stages of acute HIV infection have been described by the detection of viral particles and antibody responses; a classification termed as the Fiebig staging [28]. Immediately after transmission, there is a period of about 10 days called the eclipse phase where the viral RNA is undetectable. This period is followed by the 6 Fiebig stages of acute HIV, as outlined in Table 1.1. In Fiebig stage 1, only HIV RNA can be detected, and this is shortly followed by Fiebig stage 2 that is characterised by the detection of the p24 antigen. Stage 3 occurs approximately 30 days post-HIV acquisition and is characterised by symptoms of an acute retroviral syndrome such as fever and rash. These symptoms coincide with peak viremia [29].

Table 1.1: Fiebig stage classification of acute HIV infection.

Stage	Defining finding and/or marker	Duration, mean (range), days	
		Individual phase	Cumulative duration
Eclipse	…	10 (7–21)	10 (7–21)
I	vRNA positive	7 (5–10)	17 (13–28)
II	p24 antigen positive	5 (4–8)	22 (18–34)
III	ELISA positive	3 (2–5)	25 (22–37)
IV	Western blot positive or negative	6 (4–8)	31 (27–43)
V	Western blot positive, p31 antigen negative	70 (40–122)	101 (71–154)
VI	Western blot positive, p31 antigen positive	Open-ended	…

NOTE. ELISA, enzyme-linked immunoassay; vRNA, viral RNA.

Unlike in the eclipse phase that HIV cannot be detected, HIV RNA can be detected in stage 1 of HIV, which lasts for 7 days, and up to 17 days post-HIV acquisition. After that, the p24 antigen can be detected up to 22 days after HIV acquisition, while enzyme-linked immunoassay (ELISA) can be used to test for HIV thereafter, at least 25 days after HIV infection. Western blot, which detects HIV proteins can accurately detect HIV proteins at approximately 31 days post-infection and p31, an HIV integrase antigen, can be used to detect HIV seroconverters with high sensitivity. Adopted from [28].

Upon HIV infection, innate immune responses are quickly backed up by adaptive immune responses. Particularly, CD8+ T cell responses are responsible for bringing down the peak viremia to a stable level referred to as the viral set point. At the same time, a transient recovery of CD4+ T cell counts is observed [30] (Figure 1.2). The viral set point occurs 3 to 6 months post-infection [31] and is characterised by immune pressure selection of escape mutants and viral diversification and clinical latency [32]. The clinical latency is characterised by the absence of clinical symptoms, a gradual decrease in CD4+ T cell counts, with viral load counts generally maintained at the viral set point [33]. In the absence of antiretroviral therapy (ART), the CD4+ T cell counts decline continues, and once it passes a certain threshold, there is an exponential increase in viremia accompanied by susceptibility to opportunistic infections and results to AIDS [30, 34, 35].

The World Health Organisation (WHO) has categorized HIV/AIDS in adults into four clinical stages [36]. In clinical stage 1, patients are asymptomatic and may experience persistent lymphadenopathy, where lymph nodes are inflamed due to direct viral effects. Stage 1 may last up to 10 years before clinical stage 2 kicks in with symptoms that range from mild weight loss, recurrent respiratory infections and dermatological conditions such as oral ulcers and dermatitis. As the disease progresses, clinical signs such as moderate weight loss, prolonged unexplained diarrhoea, systemic bacterial infections such as pneumonia, meningitis set in, and these are consistent with clinical stage 3 of the disease. Additionally, pulmonary tuberculosis may be observed in some patients. In clinical stage 4, HIV wasting syndrome, pneumocystis pneumonia, extrapulmonary tuberculosis, toxoplasmosis and Kaposi's sarcoma are evident in most patients.

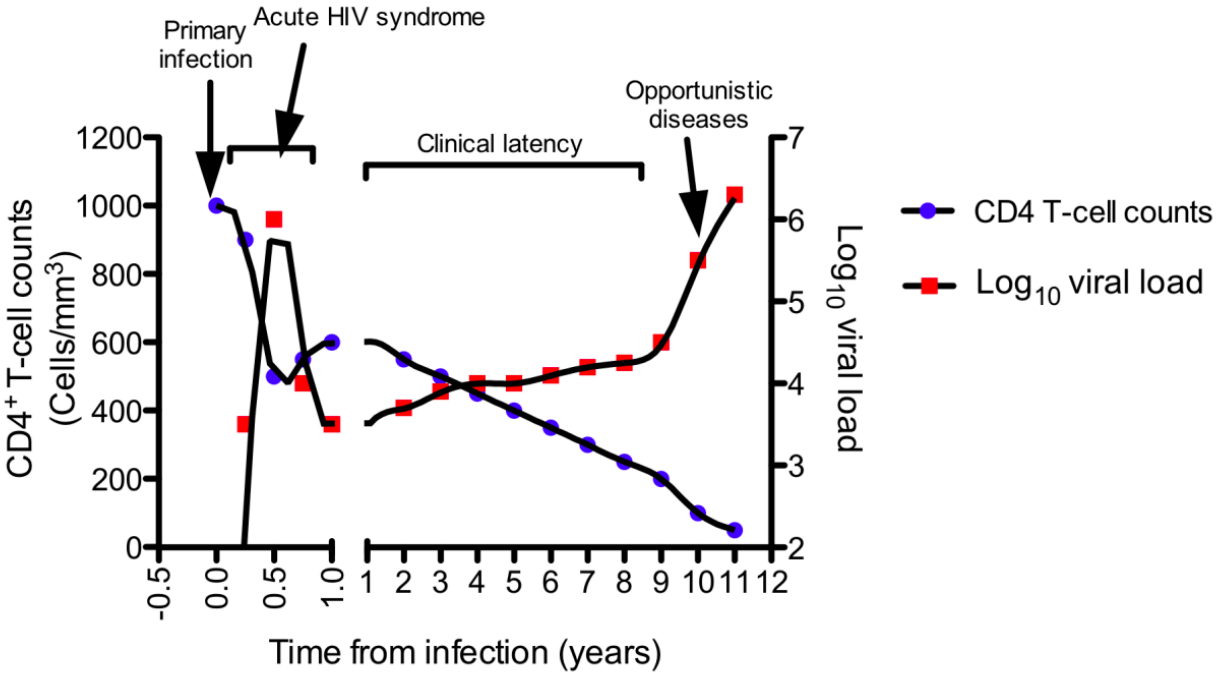


Figure 1:2: The clinical course of untreated HIV infection. Upon HIV infection, there is an exponential increase in viremia accompanied by a steep decrease in CD4+ T cell counts. CD8+ T cell responses bring down the viremia to a stable level referred to as viral set point, and there is a transient recovery of CD4+ T cell counts. However, without ART treatment, viremia gradually increases as HIV disease progresses while CD4 T cell counts gradually decrease, leading to clinical cases of opportunistic infections. The left side y-axis represents CD4+ T cell counts while the right represents Log_{10} of viral load measurements. The x-axis represents the time after HIV infection. All curves are colour coded to represent the dynamics over time of CD4+ T cell counts (blue) and HIV viral load measurements (red). Adopted from [30].

1.3: The epidemiology of HIV

According to the United Nations Programme on HIV/AIDS (UNAIDS) 2020 global HIV and AIDS report, it is approximated that up to 75.7 million [55.9 million–100 million] people have become infected with HIV since the start of the epidemic in 1980 with about 32.7 million [24.8 million–42.2 million] people dying. As at the end of 2019, HIV has infected approximately 38.0 million [31.6 million–44.5 million] of whom, 1.8 million [1.3 million–2.2 million] were children below the age of 14 years. In this period, 25.4 million [24.5 million–25.6 million] people were accessing antiretroviral therapy (ART). The incidence rates remained as high as 1.7 million [1.2 million–2.2 million] per year globally [37]. To aggravate this, the recent outbreak of the Coronavirus disease 2019 (COVID-19) collided with the HIV pandemic [38], and it remains unclear of what impact this will have in HIV infected individuals [39-41]. However, despite COVID-19 deaths being associated with immunosuppression [42], HIV infection was not found to be a risk factor for COVID-19 deaths. This is probably due to the HIV-related compromised immunity that results in a lack of intense immunological response in COVID-19 infection. Literature indicates that intense immunological responses immensely contribute to complicating the clinical course of COVID-19 [41, 43].

While the risk of acquiring HIV is significantly higher in MSMs, intravenous drug users (IDU), female sex workers and transgender people, young girls between the ages of 15-24 years are more likely to be living with HIV than men. The high HIV prevalence in girls and women is attributed to structural differences between the male and female genitalia. The high infectivity in women is because of the large mucosal surface area that is exposed to infectious fluids during intercourse. For instance, a study suggested that male to female transmission to be 1.9 (95% confidence interval 1.1 to 3.3) times more effective than female to male transmission [44].

Moreover, sociocultural factors such as younger girls having sex with older men and the subordinate position of women in society predispose women to HIV [45].

However, progress has been made in reducing new infections by adopting the UNAIDS 90-90-90 guidelines [37]. The guidelines aim at ending the AIDS pandemic by making sure that by 2020, 90% of all people living with HIV will know their HIV status, 90% of all people with diagnosed HIV infection will receive sustained ART and 90% of all people receiving ART will have viral suppression. As at the end of 2019, 81% [68–95%] of HIV infected people knew their status, and of this, approximately 82% [66–97%] were accessing ART. Of those on ART, 59% [49–69%] were virally suppressed as at the end of 2019 [37].

Furthermore, prevention of mother to child transmission (PMTCT) has had a significant contribution in reducing HIV incidence rates in infants born to HIV infected mothers, both prenatally and postnatally [46-48]. Moreover, pre-exposure prophylaxis (PrEP) [49], post-exposure prophylaxis (PEP) [50], consistent condom use, prevention of sexually transmitted infections and male medical circumcision have all had an impact in reducing HIV incidences [51]. Despite the reduction in HIV incident rates, HIV prevalence is increasing due to the effect of ART, whereby HIV infected individuals have a better prognosis and hence better survival rates [52]. The estimated numbers of children and adults living with HIV is shown in Figure 1.3.

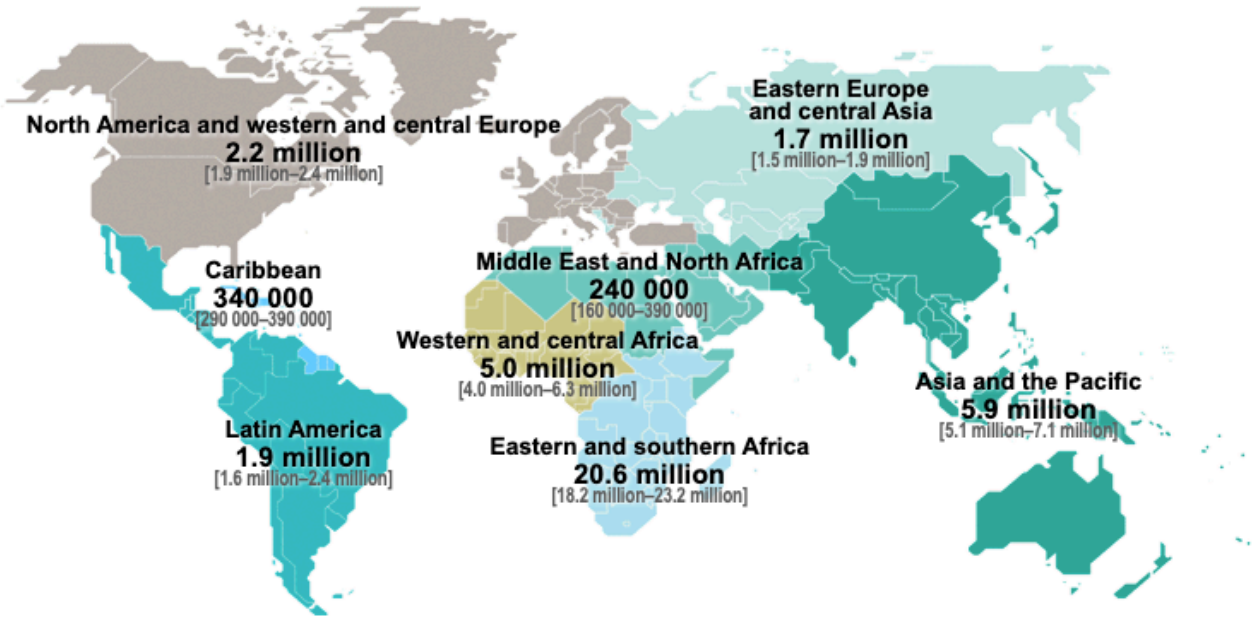


Figure 1:3: The estimated numbers of adults and children living with HIV in various countries as of 2018. Adapted from [53].

1.4: HIV antiretroviral treatments

In 2016, the WHO reviewed the guidelines on the use of ART [54] recommending lifelong ART to all HIV infected individuals regardless of their CD4 T cell count. Additionally, testing and treating of infections such as tuberculosis and cryptococcal meningitis that are associated with advanced HIV disease were officially implemented. Provision of PrEP to people at substantial risk of HIV infection was also introduced [55].

Zidovudine was the first therapy against HIV approved by the United States Food and Drug Administration (FDA) in 1987 [56]. Zidovudine is a nucleoside reverse transcriptase inhibitor that inhibits DNA synthesis by competing with the natural deoxynucleotides and terminating DNA synthesis. There are currently six FDA-licensed classes of HIV antiretroviral drugs which are classified depending on the phase of HIV replication life cycle inhibited by them. Table 1.2 highlights the classes of FDA-approved HIV antiretroviral drugs based on their mode of action [56].

Table 1.2: FDA-approved HIV antiretroviral drugs based on their mode of action [56].

Class of HIV antiretroviral drugs	**Mode of action**	**Examples of FDA-approved HIV antiretroviral drugs**
Nucleoside reverse transcriptase inhibitors	Inhibit DNA synthesis by competing with the natural deoxynucleotides	Abacavir, emtricitabine, lamivudine; Tenofovir disoproxil fumarate and zidovudine
Non-nucleoside reverse transcriptase inhibitors	Bind to the reverse transcriptase enzyme and inhibit DNA synthesis	Efavirenz, etravirine, nevirapine and rilpivirine
Protease inhibitors	Bind to the HIV protease and block the cleavage of protein precursors necessary for the production of viral particles	Atazanavir, darunavir, fosamprenavir, ritonavir, saquinavir and tipranavir
Integrase inhibitors	Prevent the insertion of viral genome into the host DNA by blocking the action of the enzyme integrase	Dolutegavir, raltegravir, elvitegravir and bictegravir
Fusion inhibitors	Prevent the fusion of HIV virions into the human cell through the disruption of membrane attachment	Enfuvirtide
CCR5 antagonists	Prevent viral attachment to the CCCR receptor on the T-cell membrane	Maraviroc

Other interventions to control the replication of HIV include post-attachment inhibitors such as ibalizumab, a monoclonal antibody that inhibits viral entry into the cell by binding to the CD4 molecule on the cell membrane. The use of pharmacokinetic enhancers has also been explored. For instance, cobicistat inhibits the human cytochrome P4503A (CYP3A) protein and thereby, increasing the concentration of other HIV antiretroviral drugs in plasma [56]. The human CYP3A protein is responsible for drug metabolism since it is involved in the oxidative biotransformation of a large proportion of drugs [57].

According to the recommendations by the WHO, the first-line ART in adults should include two nucleoside reverse-transcriptase inhibitors and a non-nucleoside reverse-transcriptase inhibitor or an integrase inhibitor except for stavudine which due to its metabolic toxicities, should not be used in the first-line regimens [55].

1.5: Immune responses to HIV

1.5.1: Early innate immune responses to HIV

Early HIV-specific innate immune responses revolve around the production of acute-phase proteins, cytokines and chemokines by innate cells. Apart from interleukin 10 (IL-10) that increases at a gradual rate, interleukin 15 (IL-15), interleukin 22 (IL-22), type I interferons and CXC-chemokine ligand 10 (CXCL10) have been shown to increase in plasma rapidly [29]. This initial cytokine and chemokine storm functions to regulate adaptive immune responses but may also have antiviral effects. For instance, type I interferon was shown to inhibit HIV replication in mice with transplanted human U937 cells, suggesting that type I interferons preserve CD4+ T cells from virus-induced damage [58].

On the other hand, prolonged exposure to cytokines and chemokines might lead to immune suppression. For example, HIV exposed dendritic cells produce indoleamine 2,3-dioxygenase, which prompts the differentiation of CD4+ T cells into T regulatory cells hence suppressing HIV-specific immune responses [59]. Moreover, findings from SIV infection suggested that natural killer (NK) cells undergo altered functions such as distorted cytokine production and homing to tissues [60], which in turn leads to inadequate immune responses.

1.5.2: Early adaptive immune responses to HIV

1.5.2.1: CD8+ T cell responses

HIV-specific CD8+ T cell responses arise slightly before peak viremia, and they play a big role in controlling viremia [15]. Responses against HIV viral peptides within proteins such as Env, Gag and Nef are among the first HIV-specific T cell adaptive responses. Indeed, HIV-specific T cell responses against Gag p24 and Pol have been reported as being important in controlling the early viremia in HIV infection through cytolysis of infected cells [61, 62]. Furthermore, there is strong evidence that individuals who lack Gag-specific CD8+ T cells tend to progress to AIDS more rapidly [63]. Similarly, the elimination of CD8+ T cells led to the dramatic increase in SIV load in macaques, translating to poor prognosis [64].

While the CD8+ T cell responses have a dramatic impact against HIV viremia initially, these responses are not sustained and rapidly decline due to exhaustion, leading to poor CD8+ T cell function. The production of perforin by CD8+ T cells significantly reduces as the disease progresses, leading to an uncontrolled increase in viremia [62]. Also, CD4+ T cells fail to secrete sufficient interleukin 2 (IL2), which translates to poor CD8+ T cell function [65]. Interleukin 2 is important for CD8+ T cell proliferation and function [65]. In support of this, in-vitro IL2 supplementation dramatically upregulates CD8+ T cell ability to lyse infected cells [65].

Even when CD8+ T cell responses are maintained, immune responses eventually lead to the selection of HIV escape mutants with altered epitopes [66], thereby abrogating immune recognition of HIV infected cells by CD8+ T cells.

There is controversy in the field on whether CD8+ T cells can traffic into B cell follicles and lyse HIV infected CD4+ T cells [67-71]. While this remains debatable, some studies have

shown that CXCR5+ CD8+ T cells exist and may play a role in controlling chronic viral infections. He and colleagues showed that CXCR5+ CD8+ T cells play a vital role in the control of chronic lymphocytic choriomeningitis virus (LCMV) in mice, as compared to CXCR5- CD8+ T cells [72]. Moreover, CXCR5+ CD8+ T cells expressed lower levels of inhibitory receptors and exhibited more potent cytotoxicity than the CXCR5− subset, highlighting their crucial role in viral replication control [72]. Similar data suggests that CXCR5+ CD8+ T cells play a role in controlling viremia in B cell follicles. Leong and colleagues conducted immunofluorescence staining of lymph node sections from both HIV infected and HIV uninfected individuals. They demonstrated significantly higher numbers of CXCR5+ CD8+ T cell in HIV infected individuals, suggesting that those cells are in the B cell follicles to lyse HIV infected CD4 T+ cells [73]. They further showed that these CXCR5+ CD8+ T cells were functional using a mouse model infected with LCMV [73]. These data suggest that CXCR5+ CD8+ T cells may play a key role in controlling HIV by lysing CXCR5+ CD4+ T cells, and this may be important in strategies designed to control and prevent HIV infection. While this uncertainty looms, a better understanding of this subset of CD8+ T cells is required.

1.5.2.2: CD4+ T cell response

HIV infects and significantly depletes memory CD4+ T cells [22]. This early depletion of CD4+ T cells can be partially reversed by administration of ART [74]. CD4+ T cells serve to coordinate the different arms of adaptive and innate immunity through the release of cytokines. Helper functions of CD4+ T cells have been studied in detail.

It has been shown that effective CD8+ T cell function squarely relies on CD4+ T cell help. CD4+ T cells in HIV infection secrete insufficient IL2, which is essential for CD8+ proliferation and function, translating to poor CD8+ T cell function [65]. More critically

studied is the help B cells receive from CD4+ T cells. CD4+ T cells offer exclusive help to B cells through cell surface co-stimulatory molecules such as CD40 ligands or cytokines [75, 76] and are vital for the development of memory B cells [76].

Also, CD4+ T cells maximize the bactericidal activity of macrophages and hence improve their immune responses [77]. Additionally, CD4+ T cells may play a role in lysing virally infected cells and have been implicated in controlling Epstein–Barr virus (EBV) [78], hepatitis C virus (HCV) [79] and HIV [80]. These cytolytic CD4+ T cells express CD107α, which enables them to have degranulation functions [80].

1.5.2.2.1: T follicular helper cell responses in HIV

T follicular helper (Tfh) cells are a CD4+ T cell subset that offers exclusive help to B cells through cell-surface co-stimulatory molecules such as CD40 ligands or cytokines [75, 76] and are vital for germinal centres (GCs) formation and the development of memory B cells [76]. They are residents of B cell follicles in GCs and are identified by their high expression of CXCR5, which enables their migration to the border of the B cell follicle and PD-1 which is an activation marker [76]. These cells can further be characterised by their expression of inducible T cell costimulator (ICOS) - a T cell activation marker, B cell lymphoma 6 protein (BCL6) - a Tfh master transcription factor, IL-21 (T cell cytokine), and CXCL13 - a marker for GCs activity and chemokine for the CXCR5 receptor present on both Tfh and B cells [81-83].

Sufficient Tfh help to B cells drives B cell proliferation, differentiation and somatic hypermutations leading to the selection of B cells with superior affinity [84]. In HIV infection, extensive levels of somatic hypermutation, that is not commonly observed in other diseases or

vaccine responses have been associated with the production of antibodies with broadly neutralising functions (bnAbs) [85].

As such, impaired Tfh cell help to B cells may be associated with reduced antibody quality as witnessed in a majority of HIV infected individuals, who fail to generate neutralising antibodies against HIV [86]. Ideally, as it is observed in other infections, adaptive immunity against HIV relies on the expansion of antigen-specific Tfh in lymphoid organs [87]. Unfortunately, HIV infects a large number of CD4+ T cell subsets, including Tfh leading to marked lymphopenia in early HIV infection and hence directly crippling the immune system.

Similarly, the abnormal differentiation of Tfh cells that is driven by viral effects may deplete Tfh cells numbers. Both HIV and SIV infections have been shown to favour the expression of T-Bet in Tfh cells leading to poor differentiation of these cells [88]. Such Tfh cells offer suboptimal help since T-Bet antagonizes IL-21. Thus, T-Bet expression by Tfh cells not only diminishes their numbers but also their function in HIV infection. This altered differentiation may also downregulate the expression of key markers such as PD-1, critical for Tfh cell function, as shown in mice models [89].

Apart from distorting the Tfh cell numbers and transcriptional profiles, HIV replication and latency phase are majorly concentrated within the Tfh population in B cell follicles, where about 60-75% of HIV-producing cells are located [90]. This is worsened by the poor penetration of ART into B cell follicles, the lack of or very low frequencies of CD8+ T cells that can traffic into B cell follicles and the presence of millions of infective virions on the follicular dendritic antigen-presenting cells [90]. The high presence of infective virions may also contribute to the expansion of HIV infected Tfh cells [91] that may further act in infecting other uninfected Tfh, and other CD4+ T cell subsets, leading to depletion of these cells. Ironically though, there are reports of increased proportions of Tfh cells in the blood of HIV

chronically-infected individuals [92], than in HIV uninfected individuals [92, 93]**.** This may be as a consequence of constant antigenic stimulation or a relative decrease in the frequency of other CD4+ T cell subsets.

The Tfh and B cell cognate interactions occur within the GCs, which are not easily accessible in most studies. The identification of a surrogate population in blood has, however advanced our knowledge of Tfh cells. Previously, human peripheral blood CXCR5+ CD4+ T cell subset has been shown to share functional properties with Tfh cells in the GCs and therefore, may be representative of the circulating Tfh memory compartment [94]. Indeed, these cells efficiently induce naïve B cells to produce immunoglobulins through their secretion of IL-21 [94]. Additionally, Havenith and colleagues suggested that the frequencies of GCs Tfh and peripheral blood CXCR5+ CD4+ T cells are associated, with the former being superior in providing help to B cells [95].

Moreover, CXCL13, a B cell follicle chemoattractant, is a marker of GCs activity [96]. Havenar-Daughton and colleagues reported a direct association between the levels of CXCL13 in plasma, the magnitude of antibody responses, the frequency of ICOS+ Tfh cells in the blood and the quality of GCs reaction [96]. The B cell activating factor (BAFF) has also been suggested to influence the survival and class switching of B cells [97] and thus may be vital in GC reactions. Since both CXCL13 and BAFF levels are readily quantifiable by ELISA in plasma, they remain essential in understanding GCs activities.

In a bid to understand basic GCs biology that may reveal immune correlates of protection in HIV, such as quality of anti-HIV antibody functions, various subsets of blood Tfh cells have been associated with bnAbs. Locci and colleagues identified blood circulating PD-1+ CXCR3- CXCR5+ CD4+ T cells to be the best subset that correlated with the generation of bnAbs in HIV [98]. Cohen, on the other hand, described global subsets of CXCR5+ PD-1+ CD4+ and

CXCR5+ PD-1+ ICOS+ CD4+ T cells in early HIV infection that positively correlated with the breadth of plasma neutralising antibody responses in chronic HIV infection [99]. Moreover, using a cross-sectional cohort of 34 untreated HIV infected controllers matched for viral load, Ranasinghe and colleagues reported a correlation of Gag-specific CD4+ T cells with antibody breadth in ART naïve HIV neutralisers [100]. Overall, current data suggest that a functional Tfh cell compartment may be vital in eliciting effective immune responses against HIV.

It is critical to note that most of the studies conducted so far have been limited in sample sizes and hence may not have provided conclusive reports. Additionally, the majority of the studies did not study HIV-specific Tfh cells responses but investigated immune responses from a global perspective. The two studies that looked at HIV-specific Tfh cell responses were cross-sectional and did not inform on the early immunological correlates that may have contributed to subsequent antibody function in chronic HIV. Although these previous studies gave a hint of potential immune correlates, it remains important to assess specific HIV immune responses as these are the cells that will offer the required specific help.

1.5.2.4: B cell responses

The bone marrow is the site of B cell development before B cells traffic to the secondary lymphoid organs for further differentiation. In the absence of ART, HIV infection causes alterations of the B cell phenotypes. Such alterations include the increase of tissue-like memory (TLM) B cells, activated memory B cells, plasmablasts and a decrease of resting memory B cells [101]. It is speculated that a large proportion of the HIV-specific B cells lie within these altered B cell subsets. Indeed, there is evidence that HIV-specific B cells may develop from immature B cell population without Tfh help and with characteristics of polyreactivity [102]. The altered B cell compartment in HIV leads to decreased quality of HIV-specific response [103] and usually translates to the poor immune response to vaccines [104].

Generally, upon antigen encounter, B and Tfh cells interact in the extra-follicular areas leading to the generation of short-lived plasmablasts. Germinal centres only form when activated B cells migrate to follicular areas that are densely packed with follicular dendritic cells (FDC), whereby, they undergo many rounds of proliferation. Only B cells with increased affinity for HIV antigens are selected and exit the GCs as memory B cells or long-lived plasma cells [105]. However, it should be noted that HIV disrupts these physiological differentiation processes by altering B cell phenotypes and frequencies, with consequences such as an increase of TLM B cells, activated memory B cells and a decrease of resting memory B cells.

The role of B cell effector function has been controversial in some studies. For instance, while antibodies have been shown to control HIV viremia [106] and SIV viremia [107], B cell depletion in rhesus macaques showed no significant difference in survival or disease progression in cases and control subjects. More interestingly, macaques whose B cells were depleted had significantly better cellular immune responses than the controls, implying that B cell depletion may have been compensated by T cell functions or that certain B cell subsets have inhibitory functions on T cells [108]. Contrarily, HIV elite controllers have been shown to have similar numbers of circulating memory B cells to those observed in HIV-naïve individuals [109] suggesting that a compact B cell compartment may be vital in controlling viremia.

While the role of B cells remains uncertain, what is known is that antibodies against HIV infection are majorly ineffective, with only 10-20% of infected individuals developing antibodies with broadly neutralising functions [110-112]. Indeed, the earliest antibody responses have been shown to target non-neutralising epitopes of the HIV Envelope, with the virus rapidly evading these responses through mutation [113, 114]. HIV-specific IgA responses at mucosal sites are generally low, despite the mucosal sites being the prevailing site of

transmission and infection [115-117]. A rapid decline in HIV-specific IgG_3 antibodies during early HIV infection has been observed and could be reflective of possible GCs destruction [118].

On their part, B cells are significantly susceptible to HIV induced hyperactivity [119]. Hyperactivation in the B cell compartment is manifested by hypergammaglobulinemia [120], elevated polyclonal B cell stimulation [120], increased frequency of B cell malignancies [121], increased B cell turnover [122], increased production of autoantibodies [123], and an increase of differentiation of B cells into plasma cells [122]. More directly, GCs B cells require IL-17 from Tfh cells in order to mature into functional memory B cells [124], and the change in the levels of IL-17 reported in HIV infection may be crucial for the inadequate B cell responses observed. Indeed, B cell dysfunction in HIV has been illustrated to occur during the cognate interaction between Tfh and B cells, where the expression of PD-L1 directly undermines Tfh help [86]. PD-L1 expression is elevated on B cells during HIV infection, and the blockage of the PD-L1/PD-1 interaction in HIV has been shown to improve IgG secretion in Tfh-B cell in vitro co-cultures [86].

1.5.2.5: Association between Tfh function with the generation of broadly neutralising antibody responses in HIV

Broadly neutralising antibodies (bnAbs) take years to develop and only occur in approximately 5-20% of HIV infected adults. These antibodies often show high somatic hypermutation [84, 125] and tend to exhibit elongated, hydrophobic complementary-determining regions (CDR) H3 residues, and autoreactivity *in vitro [123].* Additionally, few HIV infected individuals develop antibodies that can neutralise the infecting virus. However, these antibody responses lag behind a rapidly diversifying virus, rendering the antibodies ineffective to the heterologous

viral strains. Given the strong suggestions that bnAbs can control viremia in SIV [126-128], it is crucial to understand the mechanisms leading to the generation of such effective antibodies.

On the other hand, antibodies that neutralise autologous viruses take about 3 to 12 months to develop and do not inhibit heterologous viruses. More recently, bnAbs with lower hypermutation have been described in HIV infected infants after 1 year of infection, indicating a more direct path to their generation [129]. Despite the lower hypermutation, the infant neutralising activity compared with the second generation adult bnAbs. Such information provides renewed hope that neutralisation breadth does not necessarily require years of maturation and may be attainable through vaccination, although the exact pathways to achieve this remain undetermined.

HIV bnAbs are more likely to be poly- and autoreactive than anti-HIV antibodies that lack breadth [130], suggesting that this may be key in developing bnAbs. It is hypothesized that poly- and autoreactive features lead to antibody heteroligation with both host, and HIV virions, increasing anchoring targets and thus resulting in better affinity and neutralisation [130]. This is termed as neutralisation selection bias [130]. Broadly neutralising antibodies from HIV infected individuals have previously been reported to offer protection in animal models after passive intravenous and vaginal transfer [126-128]. Additionally, neutralising antibodies have been evidenced to reduce the establishment of viremia in HIV infected humanized mice [131] and delaying virus rebound in ART-interrupted HIV chronic patients [132]. These data strongly suggest that bnAbs may be a critical component of an effective HIV vaccine.

What triggers the generation of bnAbs remains unknown, however high viral load, HIV subtype, superinfection and host genotype may play a role. [96, 110]. Additionally, a robust cellular response may be beneficial in generating bnAbs. More specifically, the role of peripheral blood memory PD-1+ CXCR3- CXCR5+ CD4+ T cells in the development of

bnAbs has been highlighted in HIV, emphasizing that B cells are capable of generating functional antibodies if quality Tfh help is received [98]. In agreement, the frequencies of global CXCR5+ PD-1+ and CXCR5+ PD-1+ ICOS+ CD4 T cells in early HIV infection correlated with the breadth of plasma neutralising antibody responses in chronic HIV infection [99].

Contrarily some studies have shown a lack of correlation between the frequencies of peripheral Tfh cell in early HIV infection with antibody function in chronic HIV infection [133]. In this particular study, they reported impaired antibody production in co-cultures that was accompanied by lack of neutralisation abilities. Lindquist and colleagues used lymph node samples to characterize Tfh cells in ART naïve, and ART-treated HIV positive individuals and showed a significant expansion of Tfh cells in chronic HIV that drove the perturbation of B cell differentiation leading to poor antibody function[93]. Their observations suggest that Tfh numbers may not necessarily improve antibody generation. A better understanding of how bnAbs are generated in HIV is, therefore still needed.

1.5.2.5: Non-neutralising antibody responses

There is growing interest in the role of non-neutralising (Fc-mediated) antibodies in the control of HIV infection. Unlike Fab-mediated antibody function that takes years to develop, Fc-mediated functions are common and are induced earlier in infection [134]. Non-neutralising antibodies are capable of coating an HIV infected cell and recruit innate immune cells to kill the HIV infected cell. Such antibody functions include antibody-dependent complement deposition (ADCD), antibody-dependent cellular phagocytosis (ADCP) both which mediate the destruction of virally infected cells, antibody-dependent cell-mediated virus inhibition (ADCVI) that limits viral growth and antibody-dependent cellular cytotoxicity (ADCC) that is mediated through the recruitment of natural killer (NK) cells [135].

These functions occur through the engagement of Fcγ receptor with cellular receptors on effector cells and thereby lysing virus-infected cells [136]. Natural killer cells (NK) are the primary effector cells mediating ADCC mechanism in HIV infection, whereby, once they are activated by ADCC mechanism, they release cytotoxic granules that kill the infected cell [137]. While the ADCC mechanism may be useful in controlling viremia, dysfunction of effector cells and the presence of immune escape variants still undermines its action [136]. For example, it has been suggested that NK cells lose their ability to secrete cytokines and undergo changes on their cell-surface markers leading to defective ADCC functions that may lead to HIV disease progression [138]. Another mechanism used by HIV to evade ADCC activity is through the HIV viral protein U (Vpu), which interferes with the function of the host restriction factor tetherin and thus enhances virus spread to uninfected cells [139, 140]. Without this interference, tetherin functions in restricting HIV particles on the surface of the infected cells, which enables non-neutralising antibodies to bind to infected cells and recruit natural killer cells. Nevertheless, it has been shown that these defects may partially be reversed after successful ART [141]. Monocytes and neutrophils can mediate phagocytosis after engaging the Fc portion of antibodies.

Nevertheless, there is growing evidence that Fc effector function may play a role in controlling viremia. Various studies have described the role of ADCC in HIV infection. For instance, ADCC activity was reported to be the immune correlate of protection in the RV144 HIV vaccine trial in Thailand, where the vaccine efficacy was reported to be 31.2% [142]. Similarly, a ten-year longitudinal study involving 118 HIV infected men, classified as either non-progressors, slow progressors or rapid progressors to AIDS revealed that ADCC plays a role in viremia control [143]. The study reported that high titres of antibodies that mediated ADCC correlated with successful host defence against AIDS, and this was evident in the non-

progressors group [143]. A similar study assessing antibody responses in HIV controllers versus viremic individuals revealed significantly higher ADCC activity in the elite controllers and thus suggesting ADCC may be responsible for the low viremia [144].

Antibody-dependent cellular cytotoxicity activity has been reported in breastmilk and has directly been associated with reduced risk of mother to child transmission [145]. Perhaps even more convincing is that elite controllers demonstrated more functionally coordinated Fc-mediated responses [146]. Elite controllers were able to recruit ADCC, monocyte and neutrophil phagocytosis better than viremic individuals [146]. It is also worth noting that the Fc receptor-mediated non-neutralisation activity contributed to better broadly neutralising antibody function in the SIV macaque model. Macaques with antibodies that had deletions in both Fc receptor and complement system had poor neutralising activity when compared to macaques that either had deletions in the complement system alone or had unaltered antibody systems, highlighting the added value of Fc-mediated activity [147]. Thus, more information on cellular mechanisms that may confer better ADCC antibodies is required.

In summary, it appears that strong cellular interactions between B and Tfh cells in the GCs are crucial in an individual's ability to generate broadly neutralising antibody responses in HIV. This has been supported by various macaque studies that have shown evidence where broadly neutralising antibodies were able to control SIV viremia. Additionally, the HIV vaccine trial in Thailand together with other key studies in HIV infected individuals, macaque models and in-vitro studies strongly suggest that ADCC antibodies are crucial in the protection and viremia control in HIV and SIV. Thus, understanding basic Tfh and B cell interactions may be vital in informing us why and how good quality anti-HIV antibodies develop. This will be beneficial in informing which responses and interactions should be targeted for the development of an effective HIV vaccine development.

1.6: HIV vaccine development

Despite the efforts in the last decades, there is no licensed HIV vaccine. The biggest challenge is the high degree of antigenic variation due to the mutability of the virus [148]. Additionally, the structure of the Envelope (ENV) glycoprotein that obscures conserved viral epitopes and the instability of the trimer provides further challenges [149].

The first two trials in 1998 using bivalent subunits of the gp120 receptor binding subunit of Env aimed at inducing protective antibodies [150, 151]. One of the trials was a randomized, double-blind, placebo-controlled efficacy trial called AIDSVAX B/E (VAX 003) that tested vaccine efficacy in injection drug users[151]. The vaccine design was unsuccessful and failed to protect the vaccinees. Similarly, a vaccine with a similar design, AIDSVAX B/B (VAX 004), also failed to be protective [150]. A different vaccination approach using vectored – HIV proteins were tested in the STEP trial. The STEP trial tested the ability of adenovirus five (Ad5)-vectored internal HIV proteins (Gag, Pol, and Nef) in inducing cytotoxic CD8+ T cell responses [152]. However, the trials were stopped due to the lack of protection in vaccinated individuals [152]. Similar findings were evident in the Phambili trial that also sought to elicit T cell-mediated immune responses [153].

The only trial to show moderate efficacy was conducted in Thailand on participants that had a relatively lower risk of acquiring HIV [154]. The trial used a canary poxvirus prime (ALVAC) followed by an ALVAC + bivalent gp120 protein in alum boost to induce protection against HIV. The vaccine showed 31.2% efficacy, but this protection waned off rapidly. Similarly, the HVTN 705 trial conducted in MSMs using DNA priming and subsequent boosting with Ad5 expressing a secreted form of the Env failed to offer protection [155, 156]. Other vaccine trials have either been tested in preclinical, phase 1 or phase 2 trials with poor outcomes. For

instance, a phase 2b trial (HVTN 505) using a DNA prime-recombinant adenovirus type 5 boost (DNA/rAd5) vaccine regimen showed a lack of protection against HIV transmission [155].

More recently, HVTN 702 and HVTN 705 trials were initiated in Africa, but the former was stopped in January 2020 due to its ineffectiveness in preventing HIV transmission [157]. The HVTN 702 trial used an approach similar to the RV144 trial but replaced the clade B HIV Env proteins with HIV Env from clade C, the common clade circulating in the South African population. HVTN 705 is still ongoing and uses four Ad-26 vectored mosaic sequences followed by a trimeric gp140 Env boost as the immunogen, and initial reports have labelled it as safe, with limited side effects [149].

With there being no effective anti-HIV vaccine to date, understanding the basic biology of how quality antibody responses are generated and the cellular mechanisms leading to their production, remains key in designing an effective HIV vaccine. In this study, I sought to understand the role of HIV-specific B and Tfh-cell phenotypes on the subsequent antibody function in HIV infection.

1.7: General objective

To understand the cellular mechanisms involved in the generation of functionally relevant antibodies in chronic HIV infection.

1.8: Specific objectives

1. To characterize peripheral blood HIV-specific Tfh and B cell subsets during early HIV infection.

2. To determine HIV-specific antibody levels, isotypes and subclasses during HIV infection and as a proxy of germinal centre activity, determine the plasma levels of BAFF and CXCL13 cytokines before and during HIV infection.
3. To evaluate the quality of Fab- and Fc-mediated antibody functions in the course of HIV infection.
4. To establish if any of the early immunological events are associated with better GCs activity, antibody quality and quantities in chronic HIV infection.

1.9: Null hypothesis

Early HIV-specific Tfh and B cells do not predict the quality of downstream Fab- and Fc-mediated antibody responses in chronic HIV infection.

1.10: Justification

In the recent past, several studies have addressed the relationship between Tfh cell frequencies, phenotypes and quality with antibody breadth. However, the majority of these studies analysed general populations without demonstrating the contribution of HIV-specific subsets and function to down-stream humoral responses. Also, the majority of these studies were cross-sectional, thereby reporting immunological events at single time points and only considered neutralising antibody function.

Describing HIV-specific cell subsets and interactions may provide a true reflection of these association as these are the cells that specifically drive the HIV-specific responses. In addition, analysing these cell subsets and interactions early in the infection may be a better predictor of subsequent antibody function in chronic HIV, since antibody function is often reflective of cellular events that occurred much earlier in an infection. Often Fc-mediated functions have

been ignored despite non-neutralising activity being the only function that so far has shown an association with protection in any vaccine clinical trial, albeit being modest efficacy. This project, therefore, sought to understand HIV-specific cellular events early in an infection on the resultant antibody functions using a systems approach. The outcomes of this project will improve our understanding on the role of early HIV-specific T and B cells in the generation of relevant humoral responses downstream, information that could assist in vaccination efforts aimed at eliciting functionally relevant neutralising (Fab-mediated) and non-neutralising (Fc-mediated) antibodies.

Chapter 2 Materials and methods

2.1: Study design and participants

This study used samples from a longitudinal retrospective cohort, referred to as 'Protocol C' that was established by the International Acquired immunodeficiency syndrome (AIDS) Vaccine Initiative (IAVI). In this cohort, adults at a high-risk of acquiring human immunodeficiency virus (HIV) negative adults were recruited across different sites in Africa, that is, Uganda, Rwanda, Zambia, Kenya and South Africa and followed longitudinally. Sites in Kenya included Kenya AIDS Vaccine Initiative - Kangemi (KAVI-Kangemi); Kenya Medical Research Institute - Centre for Geographic Medicine Research-Coast (KEMRI-CGMRC) and Kenya AIDS Vaccine Initiative-Kenyatta National Hospital (KAVI-KNH).

The high-risk populations included men who have sex with men (MSMs), individuals who inject drugs (IDUs) and female sex workers (FSW) were followed up longitudinally. Upon recording a positive HIV test result either by ribonucleic acid (RNA), P24 or positive antibody test with evidence of a negative antibody test in the last 3 months, written informed consent was obtained and the individuals were enrolled into "The IAVI Protocol C cohort". An estimated date of infection (EDI) was determined based on the last negative test before the first positive test results. Quarterly follow-ups were then done for the following ten years or until a participant's CD4+ T cell counts dropped making them eligible for antiretroviral treatment (ART) as per the World Health Organisation (WHO) guidelines for HIV care at the time of the recruitment [158]. Data including demographics, HIV viral load measurements (Appendix 1) and CD4+ T cell counts (Appendix 2) were collected during the visits and have been previously described elsewhere [159]. Blood samples were collected during each of the follow-up visits and peripheral blood mononuclear cells (PBMCs) and plasma stored for future studies. This study only used PBMCs and plasma samples collected at the Kilifi site in coastal Kenya.

2.2: Sample size calculation

In a previous study, Locci and colleagues were able to identify differences in a rare subpopulation of circulating functional PD-1+ CXCR5+ CD4+ T cells in 43 HIV infected individuals with and without broadly neutralising antibodies (bnAbs) [98]. Using similar means and standard deviations as Locci and colleagues in the sample calculation, *sampsi mean 1 mean 2, (sd) (alpha) (power),* initially described by Cohen [160], an estimation with the assumptions that: alpha = 0.0500, power = 0.9000, and n2/n1 = 1.00, a sample size of n1=22 and n2=22, making a total of 44, gave a similar statistical difference for these rare subsets. Therefore, with the 53 individuals (more than the minimum required, n=44) who passed the inclusion criteria stated in Chapter 2, section 2.1, it was anticipated that this would provide enough statistical power and precision for the study.

2.3: Study Approach

With an overall aim of understanding the role of early immunological events on the subsequent antibody levels and quality, T and B cell phenotypes were determined at time point 1 (3 months PI) to determine early cellular events upon HIV infection and associated to downstream antibody levels and function. Antibody non-neutralising functions (Fc-mediated) were analysed at time points 1, 2 and 3 (3-, 12- and 48-months PI, respectively) since these are known to develop early after infection and persist throughout the infection. Antibody neutralisation function was only analysed at time point 3 (48 months PI), as these develop much later on in the infection. As antibody quantity may influence function, antibody levels and subclasses were determined at the three time points. In addition as a proxy for germinal centre (GCs) activities (BAFF and CXCL13 plasma levels as proxies) were determined at time points

1, 2 and 3 (3-, 12- and 48-months PI, respectively) and in the pre-infection plasma samples (sampled approximately 3 months before HIV infection) to understand if there are any intrinsic factors prior to HIV acquisition that influence downstream T-B cell interaction and the resulting antibody quantities and function. This also provided an opportunity to establish the kinetics of these cytokines pre- and post-HIV infection. T and B cell subsets and BAFF and CXCL13 plasma levels measured early in the infection (time point 1) were then associated with antibody functions and levels and HIV disease outcome (based on CD4+ T cell counts and HIV viral load measurements) during the chronic phase.

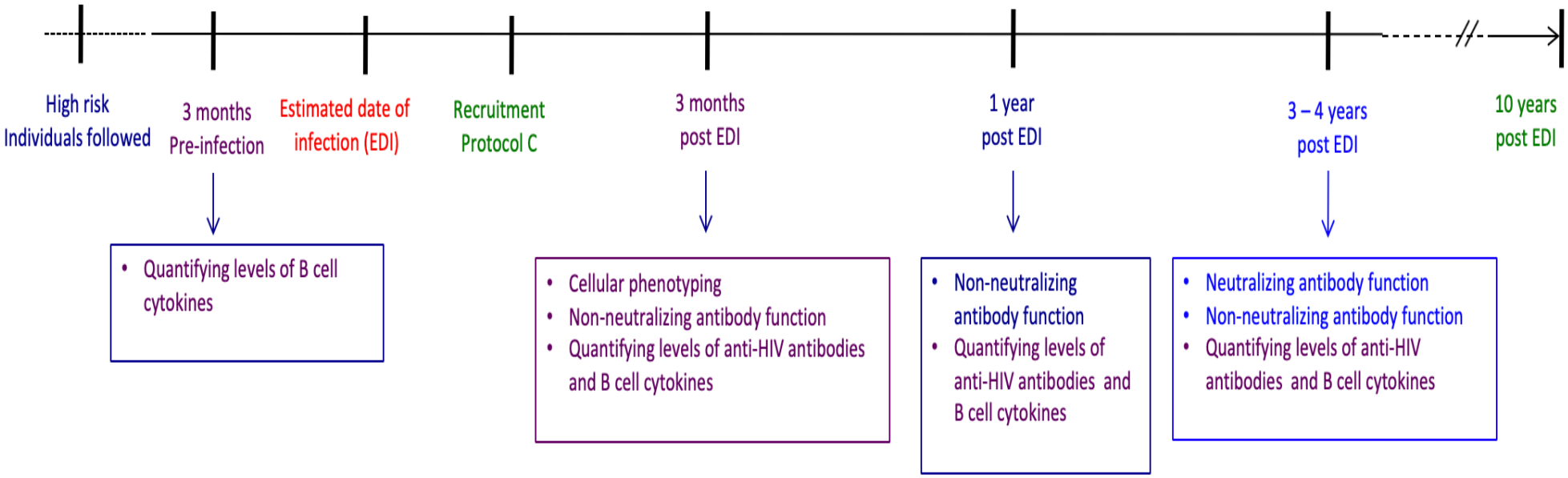


Figure 2:1: Longitudinal retrospective sampling design highlighting sampling time points when sampling and laboratory assays were done. Adults at high-risk for HIV infection were followed up every 3 months until they tested positive for HIV, at which point an estimated date of infection was determined, they were then recruited into the IAVI protocol C. Their last plasma sample to test negative before the infection was used to test for B cell cytokines levels, while their plasma samples at 3-, 12- and 48-months after HIV infection was used to run the assays as highlighted above. Additionally, PBMCs samples at 3 months PI were used for cellular phenotyping.

2.4: Ethical considerations

The study was reviewed and approved by the Kenya Medical Research Institute (KEMRI) Ethical Review Committee (ERC) under IAVI protocol C (SSC Protocol Number 1027) (Appendix 3). Written informed consent was obtained from every study participant (Appendix 4). The HIV-naïve samples were obtained under the protocol KEMRI/SERU/CGMR-C/022/3149 from adult's bleed during the annual malaria surveillance cross-sectional bleed in within Kilifi (Appendix 5).

2.5: Peripheral blood mononuclear cells sample preparation

This study used samples frozen under the above cohorts. Frozen PBMCs at approximately 10 million cells per vial were quickly thawed in a water bath at 37°C and transferred to a falcon tube containing 5 millilitres (mls) RPMI complete media (RPMI + 10% new-born bovine serum + L-glutamine + penicillin-streptomycin + hydroxyethyl piperazineethanesulfonic acid (HEPES) + β-mercaptoethanol). The cells were washed twice; each time being centrifuged at 500 g for 5 minutes at 4°C and the supernatant discarded. Consequently, the cells were counted using a Vi-CELL cell counter (Beckman Coulter) and the median cell viability was 93.05% (IQR 89.65-95.3). Four million cells were used for B cell phenotyping (general and HIV-specific subsets), 4 million cells used for the detection of HIV-specific T-follicular helper (Tfh) cells in overnight cultures and 1 million cells were used for baseline T cell phenotyping. Additionally, 200,000 cells per condition, taken from the HIV-naïve donors were used to set up the compensation panel for flow cytometry analysis and as a negative control to determine the baseline.

2.6: Characterization of peripheral blood HIV-specific and general B cell subsets during early HIV infection

2.6.1: Preparation of the B cell probe

The B cell probe, a biotin-labelled gp120 antigen, was a kind donation from Dr. Elise Landais, Scripps Research Institute. The probe was then fluorescently tagged with either streptavidin phycoerythrin (SAV PE) (Promega) or with streptavidin allophycocyanin (SAV APC) (Promega). Dual labelling of the B cells with these two probes, gp120-APC and gp120-PE, provided an optimized approach to enrich for the selection of HIV-specific B cells by minimizing non-specific selection of cells.

Briefly, B cell probes were prepared freshly for each experiment. Biotinylated gp120 antigen was incubated separately with premium grade PE-labelled streptavidin (Promega) and APC-labelled streptavidin (Promega) for at least 80 minutes on ice at a molar ratio of 4:1, to form tetramers. In order to create competition for the two-labelled streptavidin fluorochromes, the total volume of the fluorochromes to be added was divided into 8 equal volumes, and each one eighth added at intervals of 10 minutes and incubated on ice. The preparation was then mixed by vortexing and placed on ice in the dark until use.

2.6.2: Identification of B cell subsets by multiparametric flow cytometry

Approximately 4 million PBMCs to be stained for B cell phenotyping were aliquoted into a 96-well plate and washed twice using fluorescence-activated cell sorting buffer ((FACS buffer (Phosphate Buffered Saline containing 2% Foetal Calf Serum (FCS)), 5mM ethylenediaminetetraacetic acid (EDTA) and 1% Sodium Azide). To reduce the binding of gp120 probe to the preferred CD4 target in the PBMC pool, 3 µl of anti-human CD4 blocking

antibody (Biolegend) was first added and cells incubated for 30 minutes at 4°C. 2 microliters (µl) each of gp120 SAV PE and gp120 SAV APC probes that had freshly been prepared as illustrated in section 2.6.1 were then added to the cells and incubated for 30 minutes at 4°C.

A pool of the following monoclonal antibodies were then added, BV421 mouse anti-human CD19, a lineage marker for B cells, BV711 mouse anti-human CD27, a marker for memory B cells, PE-CY7 mouse anti-human CD10, a marker for B cell maturation, BV650 anti-human IgM, a marker for naïve B cells, BV605 anti-human CD20 (all from BioLegend), a marker co-expressed with CD19 and found on all B cells but plasma cells, FITC mouse anti-human CD21 (Beckman Coulter), an activation marker for B cells, Alexa Fluor 700 anti-human IgG (BD Pharmingen), a marker for B cell class switching and aqua viability dye (Life Technologies, Carlsbad, CA, USA) to exclude dead cells. The cells were incubated for a further 30 minutes at 4°C. Respective compensation control fluorochromes, including unstained cells were prepared and also incubated for 30 minutes at 4°C.

After that, 150 ul FACS buffer was added to all wells and spun at 550g for 2 minutes at 4°C. The supernatants were discarded, and 200 ul FACS buffer added to the wells and spun at 550g for 2 minutes at 4°C in order to wash off unbound antibodies. The supernatant was then discarded, and cells fixed at 4°C for 7 minutes by adding 200 ul of 1% formaldehyde. The plate was then spun at 550g for 2 minutes at 4°C, supernatant discarded; and the cells washed once in 200 ul FACS buffer. The cells were then re-suspended in 300 ul FACS buffer and acquired on the LSR Fortessa (Becton Dickinson, Franklin Lakes, NJ, USA). Data were analysed on FlowJo version 10 (FlowJo LLC, Ashland, OR, USA) and then exported to Microsoft Excel (Microsoft), GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and R Studio 1.0.143 for statistical analyses.

2.6.3: Characterization of baseline peripheral blood T cell subsets during early HIV infection

Approximately 1 million PBMCs, as described in section 2.5 above, were used for baseline T cell phenotypic characterization. The cells to be stained were plated in a 96-well plate and washed twice using FACS buffer. The following antibodies were then added, BV711 mouse anti-human CD27, a marker for T cell activation, BV421 mouse anti-human CXCR3, a chemokine receptor for T cell trafficking and function, BV605 anti-human CD45RA, a marker for naïve T cells, BV650 anti-human CCR7, a marker critical for lymphocyte trafficking into and within lymph nodes (all BioLegend), FITC mouse anti-human CD4, a co-receptor in major histocompatibility complex (MHC) class II-restricted T cell activation (Beckman Coulter), Alexa Fluor 700 anti-human CD3, a T cell co-receptor that is involved in activating both CD8+ and CD4+ naive T cells, APC anti-human CXCR5, a chemokine receptor which contributes to Tfh and B cell localization in B lymphoid follicles, PE anti-human PD-1, an inhibitory receptor that is expressed by all T cells during activation, PERCP e710 anti-human inducible T cell co-stimulator *(ICOS),* a marker for activation on T cells, (all e-Bioscience) and aqua viability dye (Life Technologies) to exclude dead cells.

Antibodies were incubated at 4°C for 30 minutes, before adding 150 ul FACS buffer to all wells and spinning the plate at 550g for 2 minutes at 4°C. The supernatant was discarded and 200 ul FACS buffer added to the plates and spun at 550g for 2 minutes at 4°C to wash off unbound antibodies. The supernatant was then discarded, and the cells fixed by adding 200 ul of 1% formaldehyde added and incubating at 4°C for 7 minutes. Plates were then spun at 550g for 2 minutes at 4°C, the supernatant discarded; and the cells washed once in 200 ul FACS buffer and spun (550g for 2 minutes, 4°C). The cells were then re-suspended in 300 ul FACS buffer and acquired on the LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA). Data were analysed on FlowJo version 10 (FlowJo LLC, Ashland, OR, USA) and then exported to

Microsoft Excel (Microsoft), GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and RStudio 1.0.143 (PBC, Boston, MA) for statistical analyses.

2.6.4: Characterization of peripheral blood HIV-specific T cell subsets in early HIV infection

To identify HIV-specific T cells, the Activation Induced Marker (AIM) assay that has been previously described [161, 162] was adapted. In summary, PBMCs are stimulated with antigen-specific peptides and the expression of the activation surface markers CD25 and OX40 measured. Both CD25 and OX40 are expressed on CD4+ T cells following T cell receptor (TCR) stimulation [161, 162]. OX40 interacts with OX40 ligand on B cells to ensure cognate T and B cell interactions while CD25 is an interleukin-2 (IL-2) receptor and important for Tfh activation [161, 162]. The AIM technique is more superior to the conventional intracellular cytokine assay, as it offers better sensitivity and specificity in detecting antigen-specific CD4+ T cells and has been reported to have significantly minimal by-stander activation [161, 162].

Peripheral blood mononuclear cells from HIV infected and uninfected individuals were tested under four conditions: RPMI complete media alone with no stimulant (as the negative control), staphylococcal enterotoxin B (SEB) (Toxin Technology, Sarasota, FL) stimulation (as the positive control), cytomegalovirus (CMV) (MyBioSource) (as another endemic virus in the study region) and the test antigen (HIV peptide pool – targeting Envelope gene) provided by the Human Immunology Laboratory, London. Each condition was set up using 1 million PBMCs. In addition, PBMCs from HIV-naïve individuals were also tested under the same conditions and used to set each compensation controls for flow cytometry analysis.

In brief, PBMCS samples were prepared as described in section 2.6.3 above. One million cells per condition were then plated into a 96 well plate and stimulated as follows:

i) RPMI complete media - no stimulant (negative control to check for background signals)
ii) Staphylococcal enterotoxin B (SEB) [1ug/ml] (positive control)
iii) HIV peptide pool [A3 ENV] (1.5ug/ml) (test antigen)
iv) Cytomegalovirus (CMV) [1.5ug/ml] (another viral antigen that study participants are likely to have been exposed to).

The cells were then incubated for 18 hours at 37°C. After the 18-hour incubation, the cells were spun at 550g for 2 minutes at 4°C and the supernatant discarded. The cells were then washed twice with 200 ul FACS buffer before being stained with the following monoclonal antibodies; BV711 conjugated anti-human CD27, BV421 mouse anti-human CXCR3, PE-CY7 mouse anti-human OX40, BV785 anti-human CD25 (all BioLegend), FITC mouse anti-human CD4 (Beckman Coulter), Alexa Fluor 700 anti-human CD3, APC-H7 anti-human CD8 (BD Pharmingen), PE anti-human PD-1, APC anti-human CXCR5, PERCP e710 anti-human ICOS (e-Bioscience) and aqua viability dye (Life Technologies, Carlsbad, CA, USA). Similarly, cells from HIV-naïve individuals were used to set compensation controls.

The cells were then incubated at 4°C for 30 minutes before washing off unbound antibodies using 200 ul FACS buffer and the supernatant discarded after centrifuging at 550g for 2 minutes. The cells were then fixed by adding 200 ul of 1% formaldehyde and incubating at 4°C for 7 minutes. Plates were then spun at 500g for 2 minutes at 4°C and the supernatant discarded. The cells were then washed once in 200 ul FACS buffer and the supernatant discarded after

centrifuging at 500g for 2 minutes. The cells were then re-suspended in 300 ul FACS buffer and acquired on the LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA).

Data were analysed on FlowJo version 10 (FlowJo LLC, Ashland, OR, USA) and then exported to Microsoft Excel (Microsoft), GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and RStudio 1.0.143 (PBC, Boston, MA) for statistical analyses.

2.7: Enzyme-linked immunosorbent assay for determination of HIV-specific antibody quantities

For HIV-specific titres, plasma samples were tested for 94UG103 Clade A (provided by E. Landais (IAVI Neutralising Antibody Centre, La Jolla, California)) specific antibodies using a standard in house enzyme-linked immunosorbent assay (ELISA) protocol [110]. In summary, Nunc MaxiSorp™ plates (Thermo Scientific) were coated with 50 ul of 1 μg/ml of 94UG103 (Clade A) antigen in PBS (50 μl/well) and incubated overnight at 4 °C. After discarding the coating antigen, plates were blocked with phosphate-buffered saline containing 0.1% Tween 20 (PBST) and 3% skim milk for 2 hours at room temperature. The block buffer was then discarded, and the plates were incubated at room temperature for 1 hour with 50 μl of test plasma sample diluted in PBST and 5% skimmed milk: 1:1000 for total IgG, 1:500 for IgG_1 and 1:100 for IgG_2, IgG_3, IgG_4, IgM and IgA.

The respective anti-human secondary antibodies conjugated to alkaline phosphatase (Sigma) were added per well and incubated for one hour at room temperature. To wash off unbound anti-human secondary antibodies, the plates were centrifuged thrice with PBST at 500g for 3 minutes. The assays were then developed by adding P-nitrophenyl phosphate (Sigma) substrate. The substrate reaction was stopped with 50 μl per well of 2 molars (M) sodium

hydroxide before being read at 405 nm in a Synergy™ plate Reader (BioTek Instruments Inc). Pooled HIV hyperimmune plasma samples were used as standards. Relative arbitrary antibody units were calculated by interpolating optical density readings to the standard curve of pooled hyper-reactive HIV positive plasma where the highest plasma concentration on the standard curve was assigned an arbitrary value of 1000 arbitrary units (AU). The HIV-specific antibody concentrations were expressed as arbitrary units after interpolation on GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) (GraphPad Software, San Diego, CA, USA). The data generated was then transformed for normalization.

2.8: Enzyme-linked immunosorbent assay for the determination of quantities of BAFF and CXCL13 cytokine levels before and during HIV infection

HIV pre-infection plasma samples (3 months pre-infection) and samples collected at approximately 3, 12- and 48-months PI were used to determine BAFF and CXCL13 levels using the Human BAFF and CXCL13 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's protocol.

Briefly, plasma samples were thawed at room temperature, spun at 500g for 5 minutes and the clear supernatant used for the assays. For both cytokines, all reagents and pre-coated plates were brought to room temperature before use. 50 µl of plasma diluted in two-fold using an assay diluent was added to each well and ran in duplicates. After a 2-hour incubation for CXCL13 and a 3-hour incubation for BAFF at room temperature, unbound soluble factors were discarded and the plates washed four times using 200 µl per well of the wash buffer. After the washes, plates were carefully inverted and blotted against a clean paper towel. The plates were

then incubated at room temperature with 200 µl per well with either human BAFF/BLyS conjugate or human BLC/BCA-1 conjugate for 1 and 2 hours respectively.

The plates were then washed 4 times, blotted on a clean paper towel and incubated with 200 µl per well of the substrate solution for 30 minutes at room temperature. The substrate reaction was then stopped by adding 50 µl per well of the stop solution. The optical density of each well was determined within 10 minutes using a Synergy™ plate Reader (BioTek Instruments Inc) set at 450 nm. Cytokines concentrations were calculated against the standard curve for respective plates and expressed in pg/ml after interpolation on GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) using the sigmoidal four-parameter logistic.

2.9: Multiplex assay for the detection of HIV-specific antibody isotypes against multiple clades

To ease the detection of HIV-specific antibodies isotypes and subclasses against multiple antigens from different clades, a multiplex microsphere bead assay was used. Briefly, Luminex MagPlex carboxylated beads (Luminex Corporation) were coupled to HIV peptides through covalent NHS-ester linkages with 1–ethyl–3–(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysulfosuccinimide (Thermo Fisher Scientific) following the manufacturer's recommendations.

HIV antigen-coupled beads (45 µl of a 20 microspheres / µl solution in 0.05% Tween and 0.1% bovine serum albumin (BSA) in PBS) were added to each well of a 384-well plate (Greinier). Plasma at 1:100 (IgG_2, IgG_3, IgG_4 and IgM), and 1:1000 (Total IgG, IgG_1) diluted in PBS were added to beads and incubated at 37 °C for 2 hours on a shaker. The coupled beads were then washed three times with, 200 µl of PBS containing 0.05% Tween and 0.1% BSA.

Consequently, individual isotypes and subclass detection reagents (Total IgM, Total IgG, IgG_1, IgG_2, IgG_3, IgG_4) conjugated to phycoerythrin (Southern Biotech) were added to the plates and incubated at room temperature for 2 hours on a shaker. The coupled beads were then washed 3 times with 200 µl PBS containing 0.05% Tween and 0.1% BSA and resuspended in 25 µl QSol Buffer (IntelliCyt) and read on an IQue Screener Plus (IntelliCyt). Each plasma sample was tested in duplicate and median fluorescence intensities (MFIs) of PE-conjugated detection agents used as the final readout. A pooled HIV hyperimmune plasma sample was used as a positive control while PBS was used as a negative control. All analyses were done on Microsoft Excel and GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

The HIV antigens used were: MN gp120 Clade B, BG505 SOSIP Clade A, JRFL gp140 Clade B, 92RW020 gp140 Clade A, IAVI22 gp120 Clade A, TV-1 gp140 Clade C, 94UG gp120 Clade A and Q461 gp120 Clade A. The flu and tetanus antigens were used as positive controls.

2.10: Fc-mediated functional assays

2.10.1: Coupling of fluosphere *NeutrAvidin* beads to biotinylated gp120 antigen

The Invitrogen, fluosphere *NeutrAvidin* (Ref 8775) was used for antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent neutrophil phagocytosis (ADNP), while Invitrogen fluosphere *NeutrAvidin* (Ref 8776) was used for antibody-dependent natural killer degranulation (ADNK) and antibody-dependent complement deposition (ADCD).

In order to coat beads, the 94UG103 (Clade A) antigen, a kind donation from Jardine's laboratory at the Harvard Medical School (Massachusetts, USA), was used to coat 1.0 um fluorescent Neutravidin beads (Liofe Technology) mentioned above, overnight at 4°C. Briefly, beads and biotinylated antigen were mixed in a ratio of 1:1 before the overnight incubation at

4°C. After the incubation, 500 ul of 0.1% BSA in PBS was used to wash off unbound beads by centrifuging at 13000 revolutions per minute (rpm) for 2 minutes. Carefully, without disturbing the pellet, the supernatant was aspirated, and the step repeated twice. A final spin without adding any 0.1% BSA in PBS was then done in order to get rid of any remaining supernatant. Finally, 0.1% BSA in PBS was used to resuspend the pellet. 1000 ul of 0.1% BSA in PBS was used to resuspend 10 ul of beads and 10ug of biotinylated antigen and the coupled antigen stored at 4°C, until use. The coupled beads were always prepared a day before the experiment and any remains were discarded after use to provide consistency in the assays.

2.10.2: Antibody-dependent cellular phagocytosis (ADCP)

All plasma samples were diluted 1:100 using RPMI complete media (RPMI + 10% new-born bovine serum + L-glutamine + penicillin Streptomycin + HEPES + β-mercaptoethanol). While using a THP-1 monocytic cell line (ATCC® TIB-202™), ADCP was performed as previously reported [163]. Briefly, the 94UG103 (Clade A) protein was used to coat 1.0 um fluorescent *NeutrAvidin* beads (Liofe Technology) overnight at 4°C as described above. The coupled beads were then incubated with plasma samples, each in duplicate, for 2 hours at 37°C in U-bottomed plates. After the incubation, plates were washed using 100 ul of RPMI complete media per well and spinning at 550g for 10 minutes. The supernatant was then discarded and 200 ul of THP-1 cells (50,000 per well) were added to each well. Plates were then incubated overnight at 37°C to allow phagocytosis of opsonized Neutravidin beads. THP-1 cells were then fixed using 200 ul per well of 4% paraformaldehyde by incubation at room temperature for 15 minutes. After the incubation, the cells were pelleted by centrifuging at 500g for 5 minutes and the supernatant discarded. After the centrifugation, 100 ul of PBS was added per well to resuspend the cells. The ability of antibodies in plasma to mediate monocytic phagocytosis was then assessed by flow cytometry on a BD LSR II flow cytometer (BD Biosciences) equipped with a high-

throughput sampler system at a standard flow rate of 2 ul per second and a mixing volume of 40 ul.

2.10.3: Antibody-dependent complement deposition (ADCD)

Antibody-dependent complement deposition was assessed by measuring the amount of complement component C3b deposited on 94UG103 (Clade A) antigen coupled with fluorescent Neutravidin beads (Liofe Technology) after incubation with plasma samples for 2 hours at 37°C. Briefly, plasma samples, in duplicates, diluted in 5% BSA in PBS at 1:50 and were incubated for 2 hours at 37°C with 94UG103 (Clade A) coupled antigen. While the incubation was ongoing, cold distilled water was used to reconstitute the low tox guinea pig complement (Cedarlane). An aliquot of complement (24 ul) was also heat inactivated at 56°c for 30 minutes. Both the heat inactivated and active complement were centrifuged at 13000 rpm, at 4°C for 5 minutes to remove debris. Once the plasma and coupled antigen incubation was done, 150 ul of PBS was added to each well on the plate and pelleted at 1000g for 5 minutes. The supernatant was then discarded before adding 196 ul of veronal buffer (Sigma) and 4 ul of the prepared complement to each well. Plates were then incubated for 20 minutes at 37°C.

During the incubation, goat anti-guinea pig C3b FITC (MP Biomed) was thawed and centrifuged at 3500g for 5 minutes to remove debris. This was then diluted at 1:100 in 5% BSA + PBS + 15mM EDTA and 50 ul of this was added to each well after the 20 minutes incubation. The plates were then incubated for a further 15 minutes in the dark, at room temperature, followed by washing by addition of 150 ul of PBS to each well and spinning at 1000g for 3 minutes. The supernatant was then flicked, and the beads were resuspended in 100 ul/well of PBS. Complement deposition was then assessed by flow cytometry on a BD LSR II flow

cytometer (BD Biosciences) equipped with high-throughput sampler system at a standard flow rate of 2 ul per second and a mixing volume of 40 ul.

2.10.4: Antibody-dependent neutrophil phagocytosis (ADNP)

Neutrophil cells were isolated from an HIV-naïve donor's blood. Briefly, fresh blood was mixed with Ammonium-Chloride-Potassium (ACK) lysis buffer (Thermo Fisher Scientific) in a ratio of 1:10 and incubated for 5 minutes at room temperature to lyse the red blood cells. Cells were then were spun down at 500g for 10 minutes, the supernatant discarded, and cells then resuspended at $2.5x10^5$ cells/ml using RPMI complete media and kept at 37°C until use.

In order to determine antibody-mediated neutrophil phagocytosis (ADNP), coupled gp120 antigen was incubated with plasma samples, diluted 1:100 in RPMI complete media for 2 hours at 37°C. After the incubation, plates were washed with 100 ul PBS per well and spun at 500g for 10 minutes. After discarding the supernatant, 50,000 neutrophils suspended in 200 ul of RPMI complete media were added per well and incubated for 1 hour at 37°C and plates then washed using PBS. To each well, 0.25 ul of the following antibodies were added: Alexa Fluor 700 anti-human CD3 (BD Pharmingen), APC-CY7 anti-human CD14 (a marker for monocytes) (BD Pharmingen) and Pacific blue anti-human CD66b (a marker for neutrophils) (Biolegend).

Additionally, 19.25 ul of PBS was added to each well and the plates incubated at room temperature for 15 minutes. After the incubation, the cells were washed using 200 ul of PBS per well and washed at 500g for 5 minutes. After that, 100 ul of 4% paraformaldehyde per well was used to fix the cells by incubation at room temperature for 15 minutes. The cells were then pelleted at 500g for 5 minutes and the supernatant discarded. The cells were then resuspended in 100 ul/well of PBS and neutrophil phagocytosis assessed by flow cytometry on a BD LSR

II flow cytometer (BD Biosciences) equipped with a high-throughput sampler system at a standard flow rate of 2 ul per second and a mixing volume of 40 ul.

2.10.5: Antibody-dependent natural killer degranulation (ADNK)

In order to assess ADNK activity, natural killer cells were first isolated from a HIV-naïve donor's blood using the RosetteSep™ Human NK Cell Enrichment Cocktail kit (STEMCELL Technologies) as per the manufacturer's protocol. Briefly, using a serological pipette, RosetteSep buffer was added to the blood in a ratio of 1:40 in a sepmate conical tube. After 15 minutes incubation at room temperature, 15 mls of histopaque 1077 (Sigma) was added to the RosetteSep treated blood and spun at 1200g for 10 minutes. The RosetteSep treated blood was then transferred to a new 50 ml conical tube and the cells spun down at 500g for 5 minutes. The supernatant was discarded, and natural killer cells resuspended in 25 ml of prewarmed RPMI complete media. **The cells were then counted and resuspended in** RPMI complete media supplemented with 0.001ug/ml IL-15 **at** $2.5x10^5$ cells/ml and rested overnight. These purified natural killer cells were used in the set up below to assess antibody-dependent natural killer cell degranulation.

To do this, ELISA plates were coated using 0.2ug/ml of gp120 antigen per well overnight. The following day, plates were washed 3 times using 200 ul of PBS per well. Plates were blocked using 200 ul/well of 5% BSA+PBS overnight at 4°C. The plates were then washed 3 times using 200 ul of PBS per well and 100ul of diluted plasma samples (1:100) added into each well and incubated at 37°C for 2 hours. After the incubation, plates were washed as described above and 200 ul of natural killer cells at a concentration of $2.5x10^5$ cells/ml added to each well. Additionally, the following antibodies/reagents were added to each well: 2.5 ul of *anti-human CD107*α Pe-cy5 (a marker for degranulation) (Sigma), 0.4 ul of brefeldin A (Sigma) and 10 ul

of golgi stop (BD Biosciences). Plates were then incubated for 5 hours at 37°C. After the incubation, cells were transferred to a V bottom plate and 1 ul/well of the following antibodies added: Alexa Fluor 700 anti-human CD3 (BD Pharmingen), PE-Cy™7 Mouse Anti-Human CD56 (a marker for NK cells) (BD Biosciences) and APC-Cy™7 Mouse Anti-Human CD16 (a marker for NK cells) (BD Biosciences). The plates were then incubated for 15 minutes at room temperature.

After the incubation, cells were pelleted through centrifuging at 500g for 5 minutes. The supernatant was discarded and 50 ul of Perm A (Life technologies) added to each well and the plates incubated for 15 minutes at room temperature. The plates were then washed with 150ul of PBS and centrifuged at 500g for 5 minutes to pellet the cells. Following the centrifugation, the following antibodies/reagents were added to each well: 1 ul of APC Mouse Anti-Human IFN-γ (BD Biosciences), 1 ul of PE-Cy™7 Mouse Anti-Human MIP-1α (Sigma) and 48 ul of Perm B (Life technologies). The plates were then incubated for 15 minutes at room temperature. Following the incubation, cells were pelleted by centrifuging at 500g for 5 minutes. Thereafter, to each well, 100 ul of PBS was added to resuspend the cells. Cells were then acquired using a BD LSR II flow cytometer (BD Biosciences) equipped with a high-throughput sampler system at a standard flow rate of 2 ul per second and a mixing volume of 40 ul. Figure 2.2 presents a summary of the methodology used in the 4 Fc-mediated assays and their readouts on analysis.

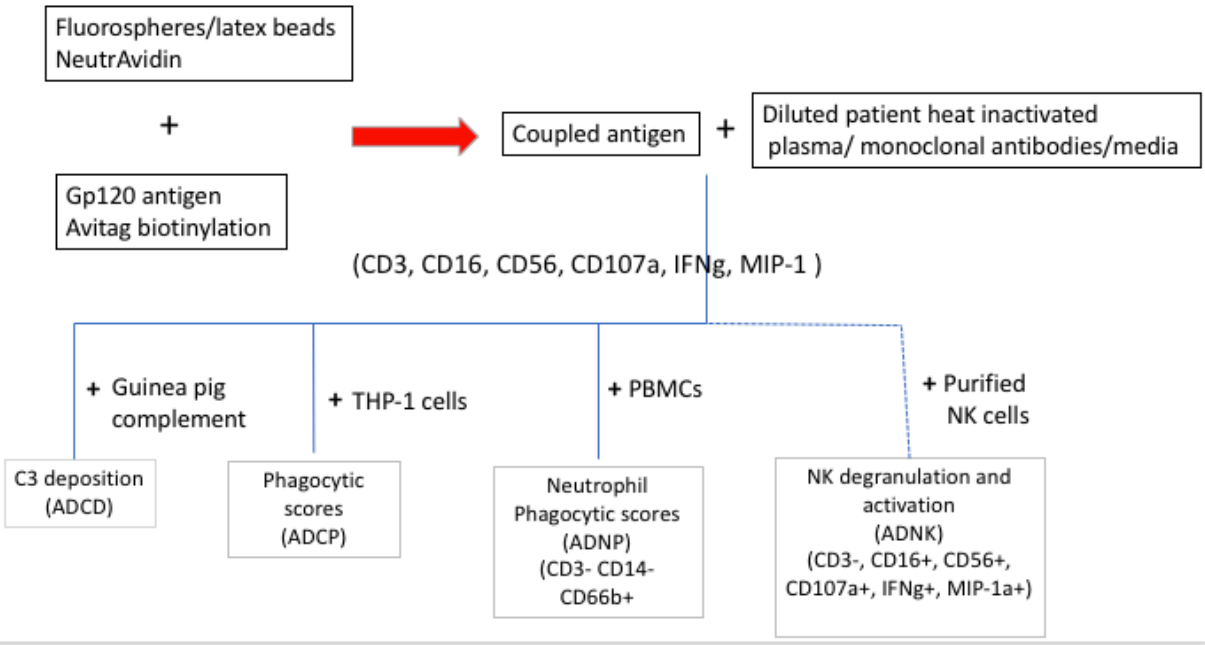


Figure 2.2: A summary of the methods and readouts used to determine the activity of ADCD, ADCP, ADNP and ADNK. Fluorescent beads were coupled to gp120 antigen and added to either plasma, monoclonal antibodies or media and incubated, after which, either guinea pig complement, THP-1 cells, PBMCs or purified NK cells was added and incubated. Scores were determined as highlighted above.

2.11: Neutralisation assay

Briefly, pseudoviruses were generated via co-transfection of 293T cells (NIH AIDS Research) with different clades of Envelope-expressing plasmid and an Envelope-deficient genomic backbone plasmid (pSG3ΔEnv) (NIH AIDS Reagent Program) in Dulbecco's Modified Eagle's Medium (Gibco BRL Life Technologies) as described previously [164]. Before producing pseudoviruses, the 293T cells were spun at 1200 rpm for 10 minutes and resuspended in 50 mls of DMEM containing 10% FBS PSG NaPyr media. The cells were then counted. For the transfection, 1600 ul of OptiMEM (Thermo Fisher), 40 ug of psG3 vector, 20 ug of the Envelope plasmid and 100 ul of Fugene (Promega) were added to a culture dish. The Envelope (Env) plasmid used were: 92BR020 (Clade B), 92TH021 (Clade AE), 93IN905 (clade C), 94UG103 (Clade A), IAVIC22 (Clade C) and JRCSF (Clade B) [165]. Murine retrovirus Envelope (MLV) and MN (B tier-1) Envelope were used as specificity and positive control,

respectively. The culture dishes were then incubated for 72 hours at 37°C. Following the incubation, the supernatant containing the pseudoviruses was collected using a pipette and stored at 37°C until use. The Fifty-percent tissue culture infective dose ($TCID_{50}$) was used to determine the pseudoviruses' titres. This dilution is used to determine the amount of pseudovirus required to kill 50% of the target cell [166].

The neutralising activity was determined as previously described [110]. Briefly, each pseudovirus was mixed with fourfold dilutions of plasma and monoclonal control antibodies starting at a dilution of 1:25. After a 1 hour incubation at 37°C, the antibody-virus mixture was added to TZM-bl indicator cell line (NIH AIDS Research) that enables analysis of HIV infectivity using luciferase as a reporter. Briefly, TZM-bl cells diluted at $0.1x10^6$ cells/mL in Dulbecco's Modified Eagle's Medium (Gibco BRL Life Technologies) were seeded in 96 well sterile plates at 50 ul/well. Plates were then incubated overnight at 37°C. The following day, pseudoviruses were harvested, and 50,000 pseudoviruses mixed with fourfold dilutions of 15 ul of plasma or monoclonal control antibodies per well, starting at a dilution of 1:25. This was then incubated at 37°C for 1 hour.

Following the incubation, 25 ul/well of the pseudoviruses and antibody/plasma mix was added to TZM-bl cells and incubated overnight at 37°C. After the 24-hour incubation, 75 ul of Dulbecco's Modified Eagle's Medium (Gibco BRL Life Technologies) was added to each well and the plates were incubated for a further 48 hours at 37°C. Thereafter, the supernatant from each well was aspirated and 40 ul of 1X lysis buffer (Promega) added to each well. The plates were then incubated at room temperature for 20 minutes. A Phelios luminometer (ATTO) was used to distribute 30 ul per well of the substrate (Promega) to each well, before reading the plates. The ability to neutralise the pseudovirus was assessed by:

$$\frac{(\text{Pseudovirus titres in wells without plasma} - \text{Pseudovirus titres in wells with plasma})}{\text{Pseudovirus titres in wells without plasma}} \times 100$$

The plasma breadth was accounted for by the number of pseudoviruses neutralised, while the potency was determined by the concentration of plasma required to neutralise the pseudoviruses. Presence of neutralising ability was defined as 50% inhibition of TZMbl cell line infection by an HIV pseudovirus. Using Microsoft Excel (Microsoft) and GraphPad Prism (GraphPad Software, San Diego, CA, USA), the infective dose (ID50) was calculated as the reciprocal dilution at which 50% of the virus was inhibited using the nonlinear regression-inhibitor versus response (3 parameters) analysis.

2.12: Data storage

All the data were stored in password protected computers, external hard disks and backed up at the KEMRI Wellcome Trust Research Programme's server.

2.13: Statistical analysis

The data were analysed using RStudio 1.0.143 (PBC, Boston, MA), Microsoft Excel (Microsoft) and GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA) softwares.

Chapter 3 Phenotypes of general and HIV-specific T and B cell subsets during early HIV infection

3.1 The biology and responses of B and T cells

The primary effector function of B cells is antibody production. However, B cells can perform other functions, including antigen presentation and cytokine production [167]. For instance, regulatory B cells (Bregs), a B cell subset that inhibits autoimmune, inflammatory and transplantation reactions, secrete high concentrations of interleukin 10 (IL-10) [168, 169].

3.1.1: B cell development in the bone marrow

The bone marrow is the primary site of B cell development before B cells traffic to the peripheral blood for further differentiation. Specifically, B cells differentiate from pluripotent hematopoietic stem cells (pHSCs) during embryonic development [170]. In foetal life, these pHSCs migrate to the liver for further development before finally trafficking to the bone marrow, where they undergo continuous development to immature B cells. In the bone marrow, B cells undergo several molecular processes that rearrange the heavy and light chains of their immunoglobulin (Ig) genes via the variable (V), diversity (D) and joining (J) (V-D-J) and (V-J) recombination processes, respectively [171]. This process is heavily dependent on interleukin-7 (IL-7), from stromal cells [172].

Two internal checkpoints confirm, first, the fitness of the IgH chain pairing with an IgL chain resulting in the formation of the pre-B cell receptor and the autoreactivity of the IgH chain of the pre-B cell receptor (BCR) leading to the formation of a BCR. Secondly, the BCR is then interrogated for autoreactivity by presenting it to different autoantigens leading to the elimination of B cells with high-affinity autoreactive BCRs. The B cells that exhibit no affinity to self-antigens exit the bone marrow, expressing a unique [173] and functional BCR and IgM or IgD [170]. Complete B cell maturation occurs in the spleen with resident B-2 type cells

remaining in the spleen while naïve B cells expressing CD19, CD20, IgM and IgD exit the spleen [170].

3.1.2: B cell differentiation in secondary lymphoid organs

Upon antigen encounter by a naïve B cell, its BCR internalizes the antigen and delivers it intracellularly to endosomal compartments [174] where antigens are degraded into peptides and loaded onto major histocompatibility complex (MHC) class II molecules and displayed on the surface of the B cell.

Other surface markers such as CD27, CD70, CD71, CD80, CD86 and CD38 are expressed [175-177]. Other cytokine receptors include CD40, CD80, IL21-r, IL6-R, IL4-r, IL10-r, interferon (IFN) -α, β, γ receptors, inducible T cell co-stimulator (ICOS) ligand and programmed cell death (PD) -1-ligand, which may play a role in modulating immune responses. For instance, CD40 is a membrane-bound receptor belonging to the tumour necrosis factor receptor superfamily that interacts with CD40 ligand on activated helper CD4+ T cells [178], leading to the formation of germinal centres (GCs). Similarly, IL-21 and its receptor (IL-21r on B cells) are essential in enhancing the proliferation and differentiation of B cells, leading to quality antibody production [174].

In human immunodeficiency virus (HIV) infection, IL-21 has been associated with HIV viremia control and better disease outcome [92, 179, 180]. Other cytokines such as interferon-gamma (IFN-γ), IL-4 and IL-6 modulate B cell proliferation and isotype class switching [181-184] while IL-10 functions in regulating B cell responses [168, 169]. Interferon alfa (IFN- α) and interferon beta (IFN-β) regulates B cell development and increased B cell receptor (BCR) sensitivity [185, 186].

Toll-like receptors (TLRs) are germline coded receptors that recognise conserved structural patterns [187] and initiate and modulate adaptive immune responses [188-190]. In human B cells, up to eight TLRs are expressed [187, 191, 192] with TLR1, TLR2, TLR5, TLR6 and TLR10 expressed on the B cell membrane, while TLR3, TLR7 and TLR9 are located in endosomes [193]. The expression of TLR1, TLR7 and TLR9 is, however lower on naïve B cells, while memory B cells subsets express high levels of TLR6, TLR7, TLR9 and TLR10 [187].

In HIV infection, there is a disruption in the B cell compartment that leads to additional B cell subsets that are not normally present in peripheral circulation (Figure 3.1). Such subsets include immature transitional B cells, activated mature B cells, exhausted tissue-like memory (TLM) B cells and short-lived plasmablasts. Expansion of these subsets during HIV infection affects other B cell subsets, such as resting memory B cells, whose frequencies are decreased [119].

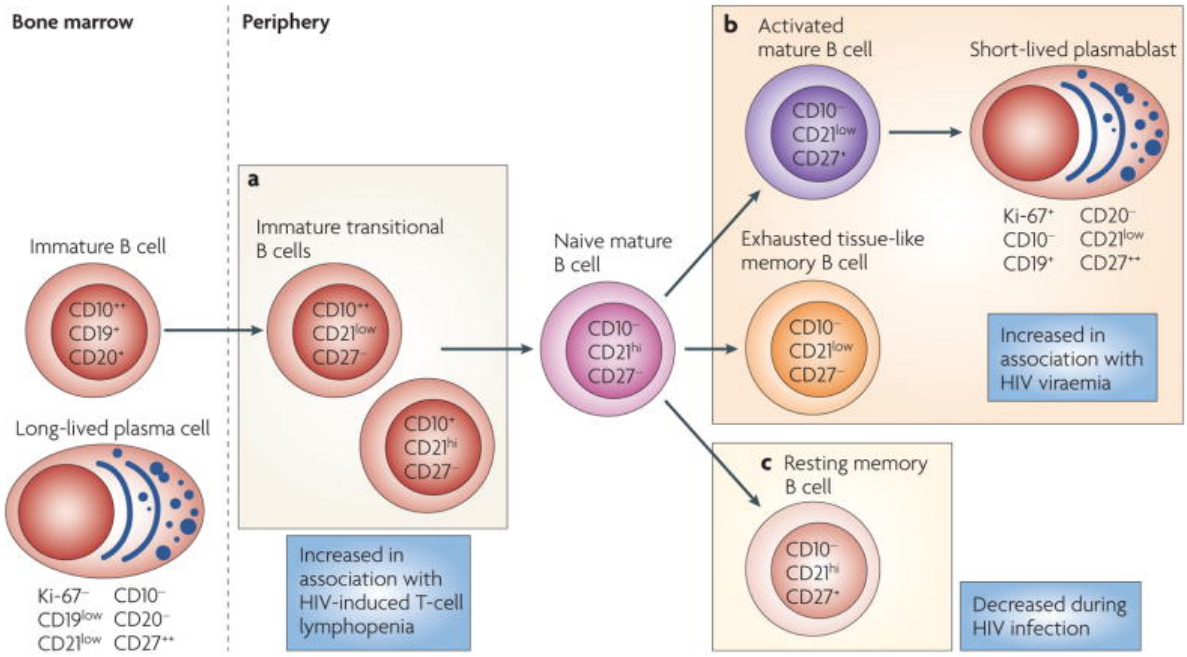


Figure 3:1: HIV-induced alterations in the B cell compartment. In principle, immature B cells expressing CD10++ CD19+ CD20+ exit the bone marrow into the periphery circulation where they express CD10- CD19+ $CD2I^{hi}$ CD27-, as naïve mature B cells. However, in HIV infection, upon exiting the bone marrow as immature B cells, they may either express CD10++ CD19+ $CD2I^{hi}$ CD27- or CD10++ CD19+ $CD2I^{low}$ CD27-, subsets referred to as immature transitional B cells (panel a). Chronic HIV is associated with the expansion of activated mature B cells that have downregulated the expression of CD21 and express CD27, short-lived Ki-67^{+} plasmablasts that have downregulated their expression of CD20 and CD21 and express high levels of CD27, and tissue-like memory B cells that have downregulated their expression of CD21 and do not express CD27 (panel b). Frequencies of resting memory B cell that express $CD2I^{hi}$ CD27+ are decreased in HIV infection (panel c). Adapted from [119].

3.1.3: T cell development in the bone marrow and thymus

Like B cells, T cells arise from a common lymphoid hematopoietic stem cell but exit the bone marrow to the thymus for maturation [194]. T cell maturation involves T cell receptor (TCR) gene rearrangement and thymocyte selection that leads to the development of a mature T cell. Like the immunoglobulin heavy and light chains, TCR α and β chains, each consist of a variable amino-terminal region and a constant region [195]. Even though the TCR gene segments rearrange in the thymus, the process is similar to the gene rearrangement in the BCR. T cell

receptor gene rearrangement involves a V_α gene segment rearranging to a J_α gene segment to create a functional V-region exon in the α chain (Figure 3.2).

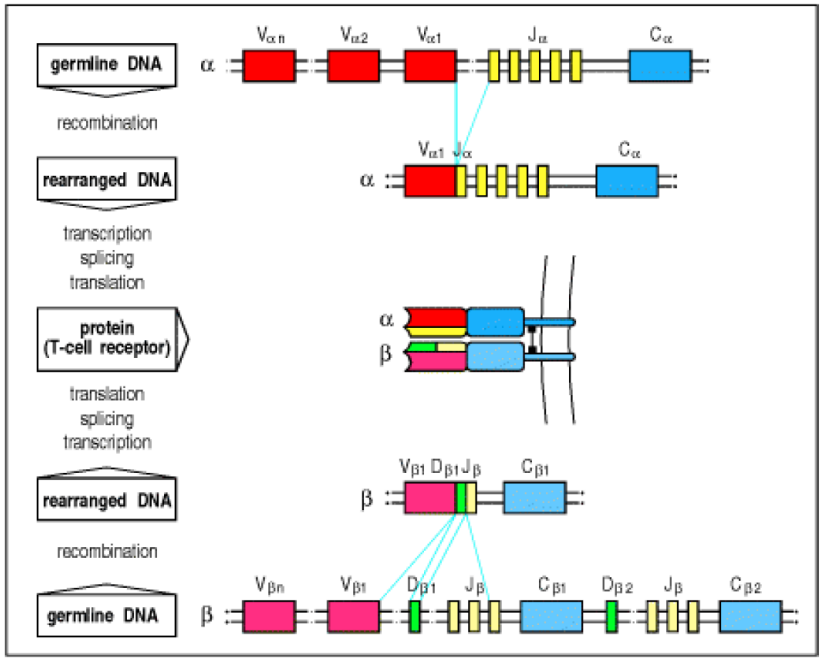


Figure 3:2: The TCR α- and β-chain gene rearrangement and expression. The TCR α- and β-chain genes comprise of discrete germline DNA segments that are joined by somatic recombination during the development of a T cell, whereby, functional α- and β-chain genes segments are generated in the same way that immunoglobulin genes are created. The α chain (upper row of the figure), is composed of a Vα gene segment that rearranges with a Jα gene segment to create a functional V-region exon. The functional VJα exon undergoes transcription and splicing and combines with a Cα gene segment and hence generating an mRNA that is translated to yield the TCR α-chain protein. Likewise, the β chain (upper row of the figure), which is encoded in three gene segments, Vβ, Dβ, and Jβ undergoes rearrangement to generate a functional VDJβ V-region exon that is transcribed and spliced to join to Cβ, resulting into mRNA that is further translated to yield the TCR β chain. The α and β chains pair soon after their biosynthesis to form the α:β TCR heterodimer (third row of the figure). Adapted from [195].

During T cell development, thymocytes migrate in the thymic microenvironments, where they come in contact with pMHC on distinct thymic antigen-presenting cells. This contact is critical for shaping the TCR for antigen recognition, the TCR thymic selection process, and the expression of surface molecules such as CD4 and CD8 [196, 197]. The thymic selection process solely relies on TCR affinity, whereby only thymocytes bearing TCR of intermediate affinity to pMHC are selected and differentiate into CD4+ and CD8+ mature T-lymphocytes

[197, 198]. Mature CD4+ T cells exit the thymus for secondary lymphoid organs where they are tasked in recognizing pMHC II molecules [199].

3.1.4: T cell differentiation in the secondary lymphoid organs

Upon interaction of TCR ($CD4^+$) with a pMHC II, CD3 activation is required to induce downstream signalling pathways, that will lead to cellular proliferation and differentiation into specific effector cells [200]. Additionally, the cytokine milieu of the microenvironment, the antigen concentration and other costimulatory molecules influence the lineage a CD4+ T cell will differentiate into [200, 201].

Of importance to this study is the T follicular helper (Tfh) cells, often described by the expression of CXCR5+ CD4+ and are known to provide help to B cells. The downstream differentiation of CD4+ T cells to Tfh cells relies on IL-6 and IL-21 cytokines [202, 203]. Upregulation of the transcription factor B cell Lymphoma 6 (BCL6) favours Tfh differentiation [75] and has been shown to inhibit the differentiation of other lineages by inducing Tfh related genes in CD4+ T cells and is thus central in the programming of Tfh cell differentiation [82]. Other transcriptional factors such as the signal transducer and activator of transcription 3 (STAT3) and c-Maf have been shown to play a role in Tfh differentiation [204, 205]. In contrast, the signal transducer and activator of transcription 5 (STAT5) were shown to be a negative regulator of Tfh differentiation [206]. Interferon regulatory factor 4 (IRF4) is necessary for the secretion of IL-21, an essential cytokine for offering B cell help and differentiation of Tfh cells [207, 208]. Also, ICOS enhancement of Tfh differentiation has been reported [209]. The peptide-MHC (pMHC)-II complexes presented by B cells can be recognised by Tfh cells that had earlier been primed by dendritic cells recognizing a

similar antigen. This cognate interaction leads to the provision of Tfh help to B cells and consequently leading to the generation of antibodies.

3.1.5: The germinal centre reaction

Germinal centres are highly specialized, localized anatomical sites in lymphoid organs, which develop in B cell follicles in response to antigenic stimulation. T-follicular helper cells traffic to the T–B border and provide the B cell help necessary for B cell differentiation into memory B cells and long-lived plasma cells.

Follicular dendritic cells (FDC), which are resident within the GCs, aid with the selection of high-affinity B cells by continuously presenting antigen on their dendrites [210]. The GCs have two zones; the dark zone where the B cells undergo several rounds of proliferation and somatic hypermutation (SHM) in the variable region of their BCR [105] and a light zone where B cells capture more antigen from the follicular dendritic cells and present them on MHC class II molecules to Tfh cells for affinity maturation and class switching [210] (Figure 3.3).

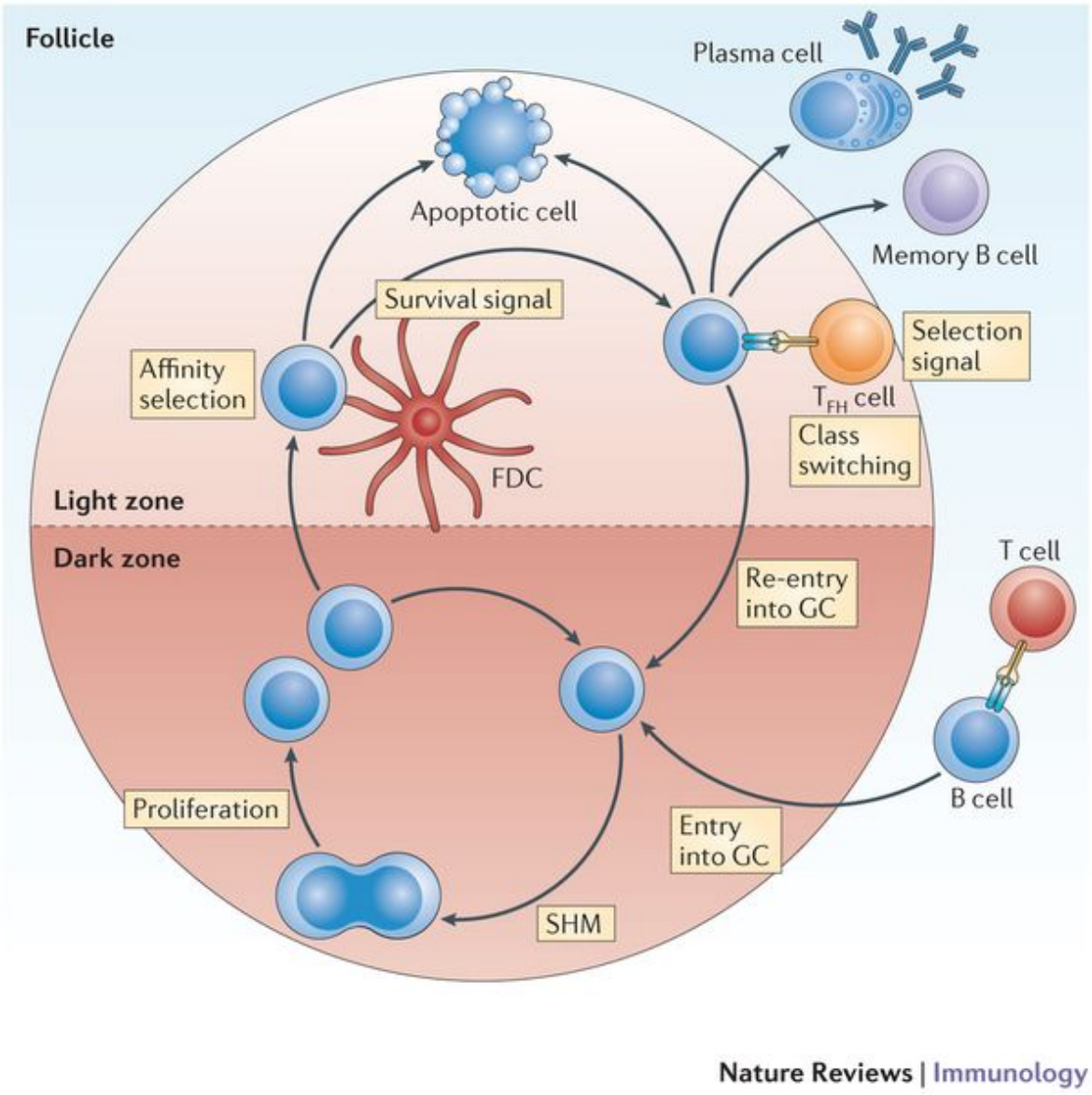


Figure 3:3: The GC reaction. The GC is divided into anatomical compartments, the dark and light zones. After the B cells present an antigen to a Tfh cell, the cells enter the dark zone of the GC where B cells undergo somatic hypermutation and may undergo one or more rounds of proliferation and somatic hypermutations. The B cells then migrate to the light zone where they are exposed to immune complexed antigens on dendritic cells for affinity maturation selection. B cells with low affinity undergo apoptosis while ones with higher affinity receive survival signals and compete for limited Tfh help. These B cells can either re-enter the dark zone and undergo further proliferation and somatic hypermutation or can exit the GC as plasma cells or memory B cells. Adapted from [211]. FDC; follicular dendritic cells, SHM; somatic hypermutation and GC; germinal centre.

3.1.5.1: Cognate interactions within the germinal centre

The cognate interaction between B and Tfh cells is dependent on the cytokines and costimulatory molecules present. For instance, CXCL13 [chemokine (C-X-C motif) ligand 13]–CXCR5 [chemokine (C-X-C motif) receptor 5] chemokine axis is vital in organizing B cell follicles and GCs [212, 213] and is responsible for the migration of CXCR5+ B and Tfh cells to the B cell follicle. The cytokine IL-21 favours the selection of high-affinity B cell clones while IL-4 enhances the high expression of CD40 ligand and directs isotype class-switch recombination [214, 215]. Other cytokines involved in the GCs reaction are shown in Figure 3.4.

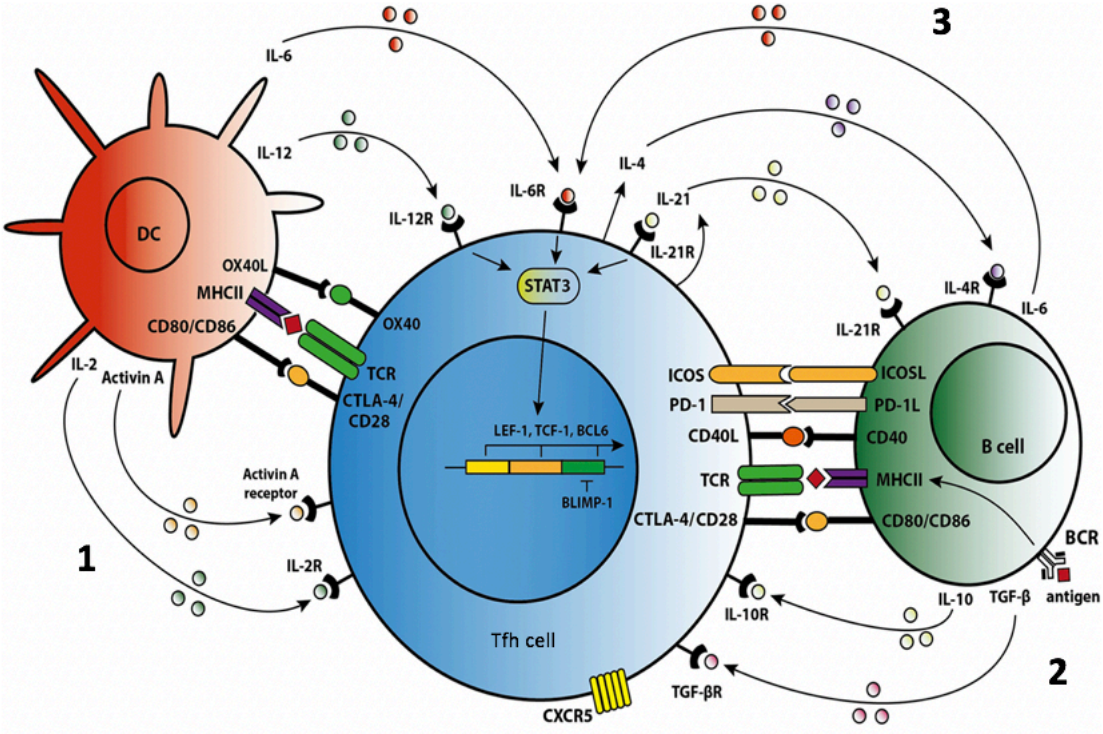


Figure 3:4: The Tfh, dendritic and B cell interaction in the GCs. (1) Dendritic cells (DC) present an antigen to a naïve CD4+ T cell through MHC II, leading to the differentiation of the naïve human $CD4^+$ T cells to the Tfh cell phenotype, a process mediated by IL-12, IL-6, and TGF-β signalling. IL-6 and IL-12 phosphorylate the transcription factors STAT3 and Activin A favour the production of IL-21 and are critical in the differentiation of Tfh cells. (2) The activated Tfh cell interacts cognately with the B cell presenting the same antigen via MHC II. The B cell contributes to the activation of the Tfh cell *via* the secretion of IL-6 and IL-10 cytokines. (3) Activated Tfh cells produce IL-21 and IL-4 cytokines that support B cell differentiation. In the dark zone of GC, IL-21 favours the selection of high-affinity B cell clones while in the light zone, IL-4 enhances isotype class switching and somatic hypermutations. STAT3; signal transducer and activator of transcription 3; IL; interleukin, CTLA-4; cluster of differentiation 154, Blimp-1; B cell lymphocyte-induced maturation protein-1, TGF; transforming growth factor, TCF; transcriptional factor and LEF; lymphoid enhancer-binding factor. Adopted from [216].

Since HIV perturbs both the Tfh and B cell compartments, I sought to characterize in peripheral blood, general and HIV-specific B and Tfh cell phenotypes during early HIV infection (3 months post infection). HIV-specific B and Tfh-cell subsets were detected using a probe targeting gp120-specific memory B cells and HIV Envelope (Env) peptide stimulation, respectively.

3.2: Objective

To characterize peripheral blood HIV-specific and total Tfh and B cell subsets during early HIV infection.

3.3: Results

3.3.1: Demographics of the study participants

Of the 98 individuals enrolled in Protocol C at the Kilifi site, 53 individuals met the inclusion criteria for this study, which was the availability of a PBMC sample at 3 months post-HIV infection (PI) and a plasma sample at 3 months PI (Time point 1), at 12 months PI (Time point 2) and at 48 months PI (Time point 3). Also, the last HIV pre-infection plasma sample, collected approximately 3 months before HIV infection, was available for each study participant. The median days before HIV infection was 91 (interquartile ranges (IQR), 72-118). As samples were not collected on the exact dates for the selected time points, a range in days before and after these time points was considered. The mean age of participants at the time of HIV infection was 24.94 [IQR 19.2-39] years. Out of the 53 recruited individuals, 48 had no more than secondary school education, and 42 belonged to the risk group of MSMs. Thirty-nine of the participants were infected with HIV subtype A, 6 with subtype C, 4 with subtype D and another 4 with the recombinant subtype AD.

Participants' clinical data, including CD4+ T cell counts and HIV viral load measurements were collected during the quarterly clinic visits and were available for analysis in the current study (Appendix 1 and 2). Table 3.1 summarises the clinical characteristics of the study population across the three time points. There was higher viremia during the first time point (3 months PI), probably indicating that the viral set point had not yet been attained. However, there were no significant differences in the median CD4+ T cell counts across the three-time

points, (Kruskal-Wallis test p=0.207) nor viral load measurements (Kruskal-Wallis test p=0.167).

Table 3.1: The clinical characteristics of study participants across the three time points, n=53.

	3 months PI	**12 months PI**	**48 months PI**	**Kruskal-Wallis test (p-value)**
Median days post-HIV infection	76 (56.25-84.72)	351 (336.5-372.5)	1030 (1008-1163)	p<0.001
Median HIV viral load (copies/ml)	38800 (8178-92300)	22750 (8223-121750)	20100 (4670-64100)	p=0.167
Median CD4+ cell counts (cells/mm^3)	508 (374-639.5)	539 9428.5-686.5)	504 (397.5-610)	p=0.207

Data shown in cells are medians (IQR). Data were collected at clinic visits at approximately 3 months PI (Time point 1), approximately at 12 months PI (Time point 2) and approximately at 48 months PI (Time point 3). There was no significant difference in HIV viral load measurements across the time points. The median CD4+ T cell counts were also similar across the three time points. p<0.05 was considered statistically significant. PI – post HIV infection. Test, Kruskal- Wallis test.

3.3.2: Gating strategy for the characterization of total and HIV-specific B cell subsets during early HIV infection

Mature B cells were defined by the expression of CD10- CD19+, class switched B cells by CD10- CD19+ CD20+ IgG+, resting memory (RM) B cells by CD10- CD19+ CD21+ CD27+, activated memory (AM) B cells by CD10- CD19+ CD21- CD27+, naïve B cells by CD10- CD19+ CD21+ CD27- and tissue-like memory (TLM) B cells by CD10- CD19+ CD21- CD27+. HIV-specific B cells were defined by gating using the gp120 probe. To reduce cross-reactivity, two fluorochromes attached to the similar antigen probe were used and the double-positive gp120 APC and gp120 PE that appear on the diagonal, gated from the switched (IgG+) B cells (Figure 3.5).

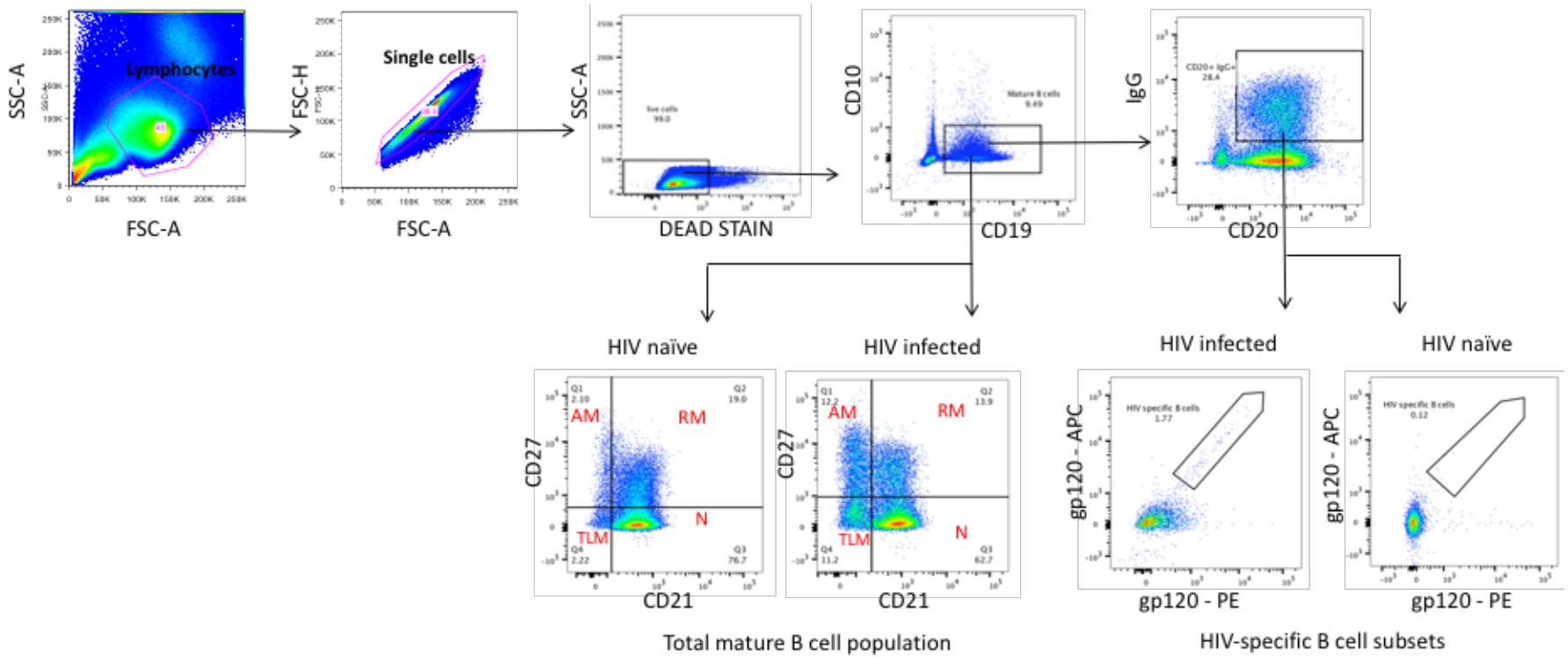


Figure 3:5: Gating strategy used to determine total and HIV-specific B cell subsets. Plots are from representative HIV infected and uninfected individuals. Mature B cells (CD19+ CD10-), class switched B cells (CD19+ CD20+ CD10- IgG+), resting memory B cells (RM) (CD19+ CD10- CD21+ CD27+) activated memory B cells (AM) (CD19+ CD10- CD21- CD27+), naïve B cells (N) (CD19+ CD10- CD21+ CD27-) and tissue like memory (TLM) (CD19+ CD10- CD21- CD27-) are presented.

3.3.3: Frequencies of total B cell subsets are altered by HIV infection

When the B cell subsets within the mature B cell compartment (CD10- CD19+) were considered, HIV-naïve individuals had a significantly higher proportion of naïve B cells (CD10- CD19+ CD21+ CD27-) 54.3% [IQR, 47.1%-65.85%] compared to the HIV infected 46.6% [IQR, 29.45%-56.25%], Mann-Whitney test p =0.0051. There was a significant increase in activated memory B cells (CD19+ CD10- CD21- CD27+), in HIV infected individuals, 12% [IQR, 6.65-21] versus 4.1% [1.7-7.7] in HIV-naïve individuals, Mann-Whitney, test p<0.0001.

Similarly, TLM B cells (CD19+ CD10- CD21- CD27-) were significantly expanded in HIV infected individuals, 20% [IQR, 12.15-29.55] compared to the negative controls 5.4% [IQR, 3.45-6.67], Mann-Whitney test, p<0.0001 (Figure 3.6). However, the resting memory B cells (CD19+ CD10- CD21+ CD27+) population was significantly higher in HIV-naïve individuals 36.8% [IQR, 26.6%-40.35%] versus 18% [IQR, 9.64-24.3] in HIV infected individuals, Mann-Whitney test p<0.0001. These findings imply that HIV infection contributes to the overall depletion of RM B cells while driving the expansion of other B cell subsets such as TLM and AM B cells that are not commonly found in peripheral circulation in a healthy state.

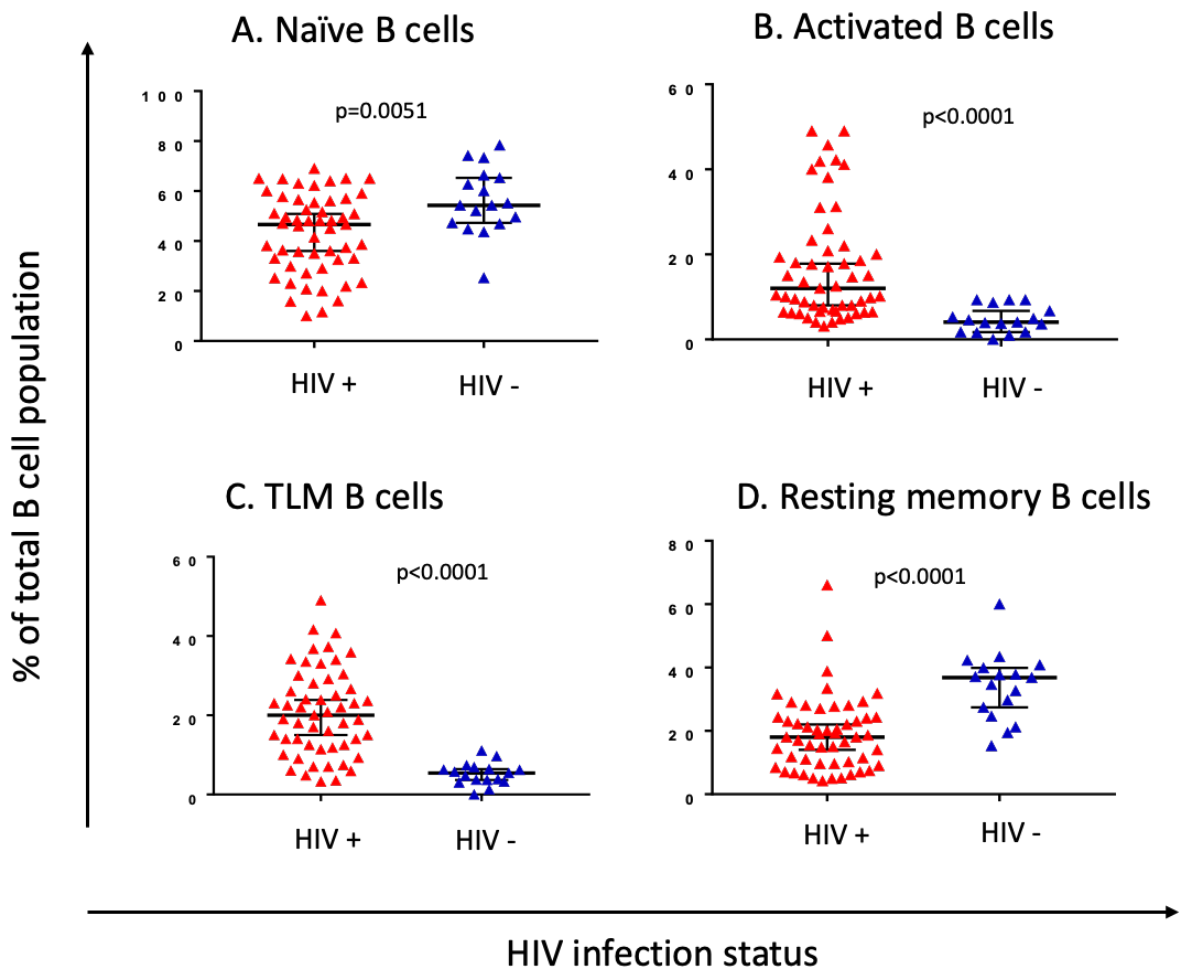


Figure 3:6: Altered frequencies of B cell subsets in early HIV infection. Each triangle represents a study participant. Y-axis; % of B cell subsets in the mature B cell population (CD19+ CD10-), x-axis; HIV infection status. Panels A, B, C and D represents naïve, activated, TLM and resting memory B cell percentages respectively, in HIV infected and uninfected individuals. Tissue like memory; (TLM). The median and interquartile ranges (IQRs) are indicated. $p<0.05$ is considered statistically significant. Mann-Whitney test was used to determine the difference between the two group medians.

3.3.4: Proportions of HIV-specific B cells

When HIV-specific B cells were considered, a median proportion of 0.58% (IQR, 0.41%-0.9%) of HIV-specific B cells was observed in HIV infected individuals. These frequencies were significantly higher than the background signals in HIV-naïve individuals; median 0.12% (IQR, 0.02%-0.22%), Mann-Whitney test $p<0.0001$ (Figure 3.7). In 8 individuals, very low

proportions of the HIV-specific B cells were identified that were similar to the background signal.

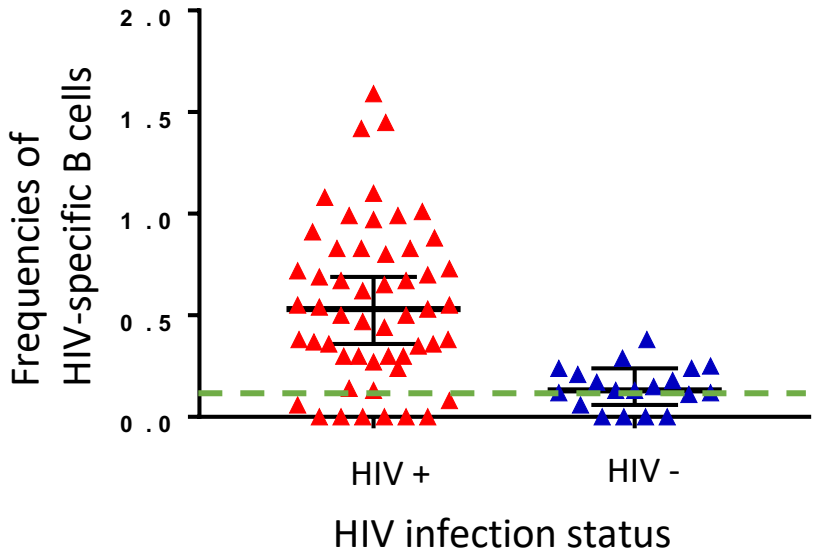


Figure 3:7: Identification of HIV gp120-specific memory class-switched B cells in HIV infected individuals. Each triangle represents an individual; y-axis shows the frequencies (%) of HIV-specific B cells in the memory class-switched B cells CD19+ CD10- CD20+ IgG+, x-axis; represents HIV infection status, y-axis. The horizontal green dotted line represents median of the background signal as determined by the HIV-naïve individuals (blue triangles). The median and IQR are indicated. Mann-Whitney test was used to determine the difference between the two group medians.

3.3.5: Association between B cell subsets and clinical parameters

Since HIV infection has been shown to drive various B cell defects [217, 218], I assessed for the correlations between the frequencies of various B cell subsets with CD4+ T cell counts and HIV viral load measurements; two measures often used to assess HIV disease progression.

No association was found between B cell subsets determined at 3 months PI with CD4+ T cell counts at 3, 12- and 48-months PI. However, when B cell subsets determined at 3 months PI were associated with HIV viral load measurements at 3, 12- and 48-months PI, both TLM B cells and mature B cell subsets showed significant associations with HIV viral load measurements. Tissue-like memory B cells were significantly associated with HIV viral load

measurements at 48 months PI and this may suggest a role of HIV viremia in driving B cell defects. However, mature B cells negatively correlated with HIV viral load measurements at 3 months PI and this may be an indication of an intact B cell compartment earlier in the infection, in individuals with lower viremia (Table 3.2).

Table 3.2: Association of B cell subsets determined at 3 months post-HIV infection with markers of HIV disease progression over time.

	CD4+ T cell counts			*Viral loads measurements*		
	3 mths PI Rho (p-value)	***12 mths PI Rho (p-value)***	***48 mths PI Rho (p-value)***	***3 mths PI Rho([p-value)***	***12 mths PI Rho (p-value)***	***48 mths PI Rho (p-value)***
Mature B cells	0.027 (0.846)	-0.063 (0.652)	0.087 (0.631)	**-0.280 (0.040)**	-0.081 (0.568)	0.000 (0.774)
Class switched B cells	0.005 (0.970)	-0.204 (0.143)	-0.206 (0.233)	0.165 (0.236)	0.313 (0.023)	0.264 (0.264)
Tissue-like memory B cells	-0.076 (0.563)	-0.029 (0.834)	-0.116 (0.356)	-0.222 (0.109)	0.054 (0.702)	**0.320 (0.019)**
Naïve B cells	0.031 (0.823)	0.1094 (0.435)	-0.015 (0.825)	-0.171 (0.221)	-0.205 (0.146)	0.001 (0.814)
Activated B cells	-0.021 (0.876)	-0.053 (0.703)	0.123 (0.251)	0.248 (0.071)	0.145 (0.303)	-0.157 (0.508)
Resting B cells	0.211 (0.174)	0.952 (0.176)	0.197 (0.252)	0.23 (0.097)	0.041 (0.974)	0.168 (0.557)
HIV-specific B cells	0.041 (0.769)	-0.102 (0.464)	0.120 (0.673)	0.114 (0.414)	0.029 (0.835)	-0.039 (0.975)

CD4+ T cell counts and viral load measured at 3, 12 and 48 months (mths) post infection were associated with B cell subsets determined early in the HIV infection (3 months PI). In each cell, the Spearman's rho value and the associated p-value is shown. Bold blue indicates significant associations. Test; Spearman's non-parametric correlation, PI; post-HIV infection. $P<0.05$ is considered statistically significant.

3.3.6: Characterization of general T cell subsets in early HIV infection

The following gating strategy was used to define general T cell subsets in peripheral circulation in early HIV infection (Figure 3.8). T helper cells were defined by expression of CD3+ CD4+, naïve T helper cells by CD3+ CD4+ CD45RA+, memory T helper cells by CD3+ CD4+ CD45RA-, memory blood Tfh cells by CD3+ CD4+ CD45RA- CXCR5+, memory blood Tfh cells expressing activation markers PD-1 and ICOS by CD3+ CD4+ CD45RA- CXCR5+

ICOS+ PD1+, central memory T helper cells by CD3+ CD4+ CD45RA- CD27+ CCR7+ and effector memory T helper cells by CD3+ CD4+ CD45RA- CD27- CCR7-.

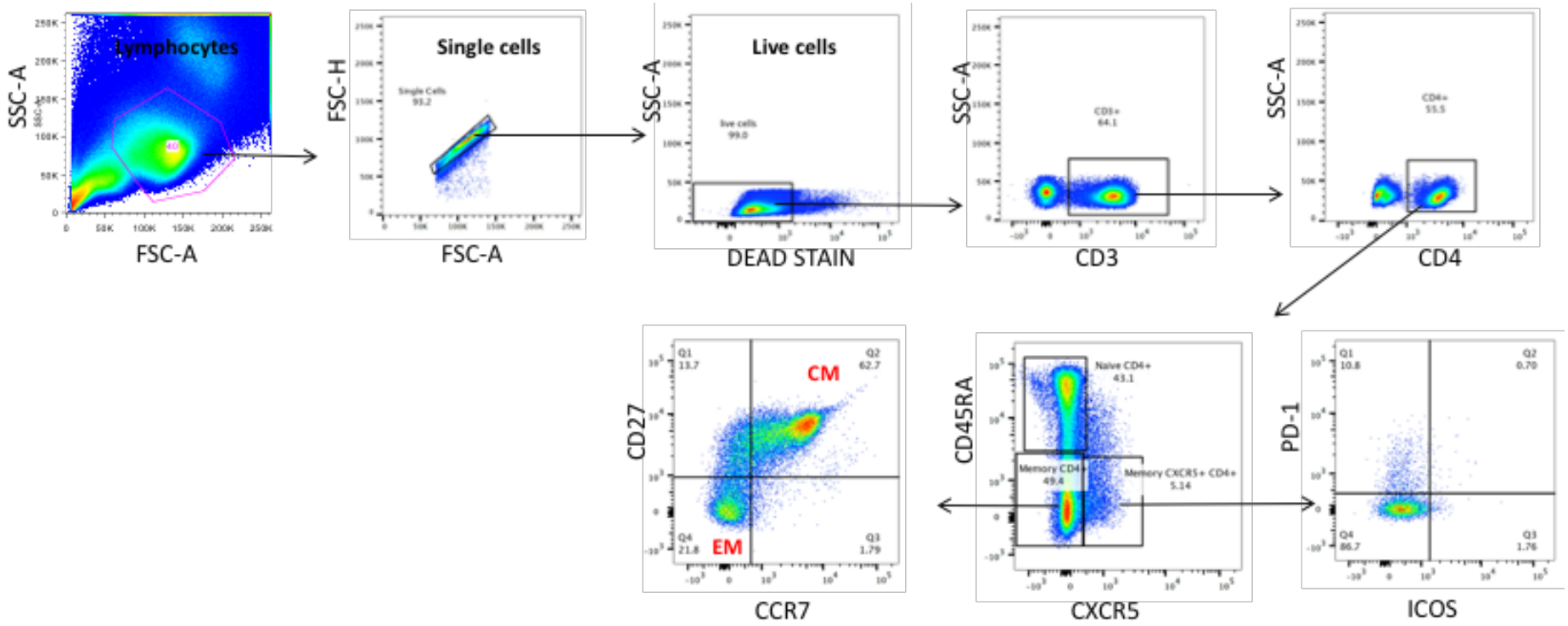


Figure 3:8: Gating strategy used to determine general T cell subsets. Plots are from a representative HIV infected individual. T helper cells are defined by expression of CD3+ CD4+, naïve T helper cells by CD3+ CD4+ CD45RA+, memory T helper cells by CD3+ CD4+ CD45RA-, memory blood Tfh cells by CD3+ CD4+ CD45RA- CXCR5+, central memory T helper cells by CD3+ CD4+ CD45RA- CD27+ CCR7+ and effector memory T helper cells by CD3+ CD4+ CD45RA- CD27- CCR7- are presented. CM= Central memory, EM= Effector memory.

3.3.7: Frequencies of general T cell subsets are altered in HIV infection

A significant depletion of CD4+ T cells and CD4+ effector memory T cells (CD27- CCR7- CD4+) and an expansion of CD4+ central memory T cells (CD27+ CCR7+ CD4+) in early HIV infection was observed (Table 3.3). The proportion of Tfh cells (CD45RA- CXCR5+ CD4+) was expanded in HIV infected individuals. Both the expansion and depletion of Tfh cells have been reported in HIV [86, 219]. A significant expansion in CD4+ cellular subsets expressing PD-1, which represents the activated cellular subsets, CD45RA- CXCR5+ PD-1+ CD4+ and CD45RA- PD-1+ CD4+ was observed in HIV infected individuals.

Table 3.3: Key CD4+ T cells are significantly depleted in early HIV infection.

	HIV infected (% of total CD4 T cells, unless otherwise stated)	**HIV naïve (% of total CD4 T cells, unless otherwise stated)**	**p-value**
CD4+ (% of total CD3+ lymphocytes)	41.6 (32.2-53.1)	50.4 (48.1-55.65)	0.004
CD45RA+ CD4+	41.88 (21.6-54)	46.38 (38-51.6)	NS
CD45RA- PD-1+ CD4+	9.5 (4.7-19.8)	5.01 (3.1-9.9)	0.002
CD27+ CCR7+ CD4+	67.4 (58.7-74.8)	47.43 (34.3-66.7)	0.004
CD27- CCR7- CD4+	16.71 (9.04-35.5)	31.11 (16.5-56.8)	0.005
CD45RA- CXCR5+ CD4+	7.6 (5.7-10.6)	6.18 (4.1-8)	NS
CD45RA- CXCR5+ PD1+ CD4+	1.87 (0.4-5.31)	0.72 (0.28-1.97)	0.021

The frequencies of CD4+ T cell subsets determined early in the HIV infection (3 months PI) and compared between HIV infected and HIV-naïve individuals. Medians and with their associated IQRs and p-values are shown. NS; not significant. (Mann-Whitney test).

3.3.8: Detection of HIV-specific Tfh cells in early HIV infection

The gating strategy presented in Figure 3.9 below was used to define HIV-specific Tfh cells in early HIV infection. T helper cells were defined by the expression of CD3+ CD4+, memory blood Tfh cells by CD3+ CD4+ CD45RA- CXCR5+, and HIV-specific, Cytomegalovirus (CMV)-specific, and Staphylococcal enterotoxin B (SEB)-activated Tfh cells by CD3+ CD4+ CD45RA- CXCR5+ CD25+ OX40+ expression after respective stimulations as described in chapter 2 subsection 6.4.

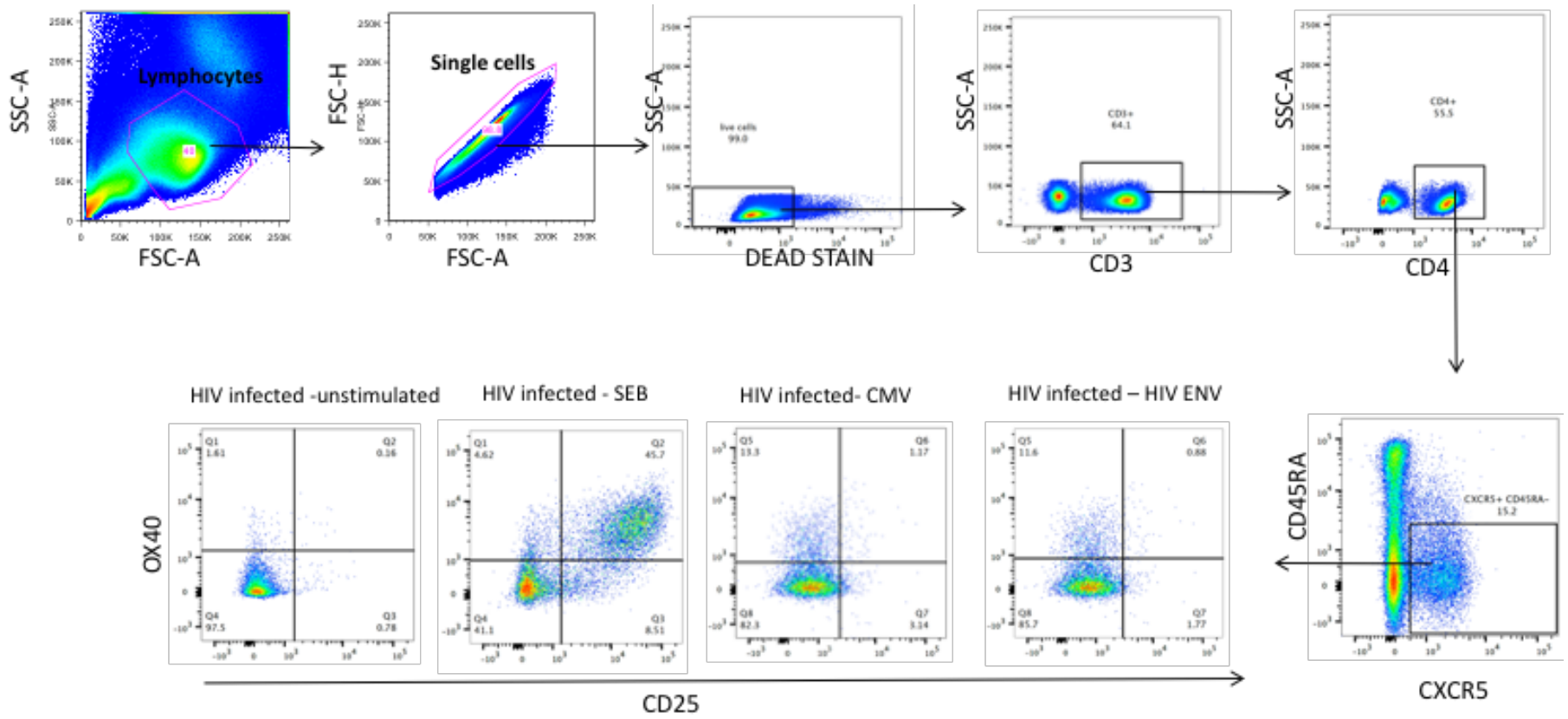


Figure 3:9: Gating strategy used to determine antigen-specific Tfh subsets. Plots are from a representative HIV infected individual. T helper cells are defined by the expression of CD3+CD4+, memory blood Tfh cells by CD3+CD4+CD45RA-CXCR5+, and HIV-specific, CMV-specific and SEB-activated Tfh cells by CD3+CD4+CD45RA-CXCR5+CD25+OX40+ expression after respective stimulations.

The presence of HIV-specific Tfh cells was defined by the upregulation of CD25+ and OX40+ on memory CXCR5+ CD4+ T cells after an 18-hour stimulation with HIV envelop peptides, while SEB and CMV peptides were used to assess for non-specific cellular responses to another virus other than HIV, respectively.

HIV infected individuals had an increased proportion of Tfh cells after stimulation with HIV peptides, 0.54% (IQR, 0.3-0.83) having subtracted the background signal in the HIV-naïve individuals (0.13%, IQR, 0.06-0.24), Mann-Whitney test $p<0.0001$. The median frequencies of CMV-specific memory Tfh cells in HIV-naïve and infected individuals were statistically similar, suggesting that one does not lose CMV-specific memory Tfh cells after exposure to HIV, [0.68% (IQR, 0.49-1.13) in HIV infected versus 0.9% (IQR, 0.5-1.7)] in HIV-naïve individuals. Similarly, in unstimulated conditions, no differences were observed in Tfh frequencies between HIV infected individuals and HIV-naïve individuals, 0.46% [IQR, 0.295-0.78] versus 0.35% [IQR, 0.22-0.78], respectively. Responses to SEB were also the similar, irrespective of an individual's HIV status; 21.8% [IQR, 15.9-33.4] in HIV infected versus 17% [IQR, 13.5-23], $p=0.44$ (Figure 3.10).

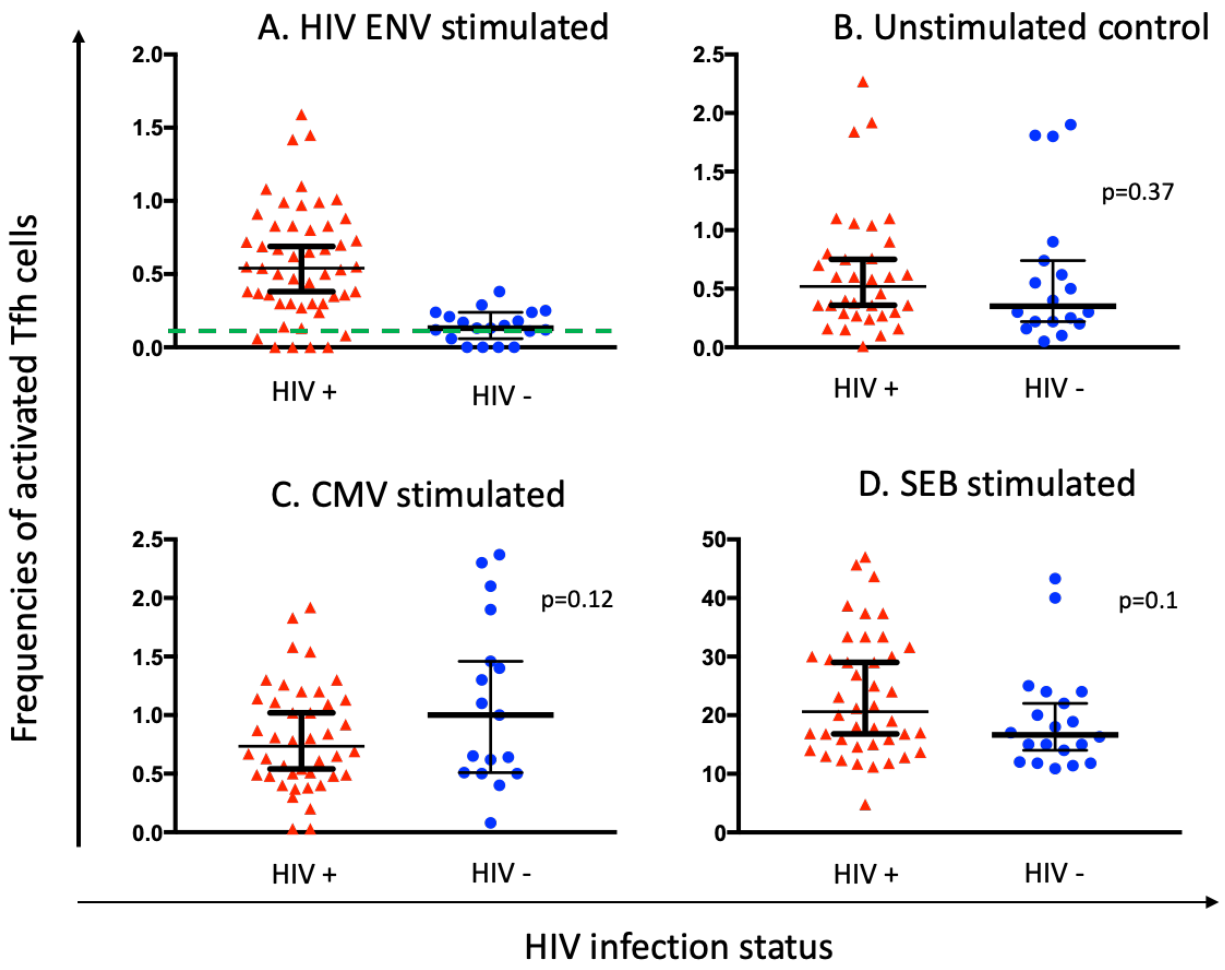


Figure 3:10: Activated memory Tfh cells (CD3+ CD4+ CD45RA- CXCR5+ CD25+ OX40+) in HIV infected and HIV-naïve individuals. HIV-specific memory Tfh cells (A) were detected after stimulating PBMCs with an HIV Envelope peptide while the controls were; no stimulation (B), CMV peptide stimulation (C) and SEB stimulation (D). Each dot represents an individual; the y-axis shows the percentage frequency of activated Tfh cells while the x-axis represents the HIV infection status. The horizontal green dotted line in panel A represents the background signal as determined by the cellular frequencies in HIV-naïve individuals (blue dots). The medians and IQR are indicated. (Mann-Whitney test.

3.3.9: Association between T cell subsets and HIV clinical parameters

Since HIV directly impacts on the CD4+ T cell population, correlations between the frequencies of various T cell subsets and markers of HIV disease progression represented by HIV viral load measurements and CD4+ T cell counts were determined. The CD4+ T cell subsets determined at 3 months PI were associated with CD4+ T cell counts and viral load measurements at 3-,12- and 48 months PI (Table 3.4). Only ICOS+ PD-1+ CXCR5+ CD4+ T cells negatively correlated with CD4+ T cell counts at 3 months PI and with HIV viral load measurements at 12 months, suggesting a role of HIV viremia in activating Tfh cells, while depleting CD4+ T cell counts.

Table 3.4: Association of T cell subsets determined at 3 months PI with markers of HIV disease progression over time.

	CD4 counts			***Viral loads measurements***		
	3mths PI Rho (p-value)	***12 mths PI Rho (p-value)***	***48 mths PI Rho (p-value)***	***3 mths PI Rho (p-value)***	***12 mths PI Rho (p-value)***	***48 mths PI Rho (p-value)***
CD4+	0.179 (0.206)	0.225 (0.112)	0.106 (0.531)	0.125 (0.125)	0.176 (0.176)	0.237 (0.412)
CD45RA- CXCR5+ CD4+	0.078 (0.956)	-0.180 (0.206)	-0.218 (0.144)	0129 (0.386)	-0.016 (0.915)	-0.059 (0.692)
PD-1+ CXCR3- CXCR5+ CD4+	0.190 (0.181)	-0.021 (0.871)	-0.054 (0.273)	0.091 (0.524)	0.099 (0.492)	0.137 (0.359)
ICOS+ PD-1+ CXCR5+ CD4+	**-0.328 (0.018)**	-0.081 (0.578)	0.047 (0.600)	0.0910 (0.525)	**-0.374 p=0.008**	-0.017 (0.909)
CD27+ CCR7+ CD4+	0.126 (0.378)	0.290 (0.39)	0.058 (0.081)	0.235 (0.096)	0.101 (0.484)	0.067 (0.090)
CD27- CCR7- CD4+	-0.043 (0.763)	-0.175 (0.219)	0.023 (0.306)	-0.165 (0.246)	-0.075 (0.603)	-0.0033 (0.321)
HIV-specific Tfh cells	-0.025 (0.861)	0.062 (0.665)	0.036 (0.847)	-0.090 (0.529)	-0.157 (0.278)	-0.204 (0.685)

CD4+ T cell counts and viral load measured at 3,12 and 48 months (mths) post infection were associated with T cell subsets determined early in the HIV infection (month 3). In each cell, the Spearman's rho value and the associated p-value are shown. Bold blue indicates significant associations. Test; Spearman's non-parametric correlation, PI; post infection. P<0.05 was considered statistically significant.

3.3.10: B and T cell interactions in early HIV disease

Since B cells receive help from Tfh cells in the GCs, associations between various total and HIV-specific B and T cell subsets populations were investigated (Table 3.5). Notably, CD4+ T cells positively correlated with some of the B cell subsets but not with class-switched memory B cells, HIV-specific B cells and TLM B cells. These positive associations with mature, naïve, AM and RM B cell subsets may suggest significant CD4+ T cell help to B cells.

Effector memory CD4+ T cells (CD27- CCR7- CD4+) positively correlated with RM B cells and negatively with TLM B cells, probably due to direct and indirect effects of HIV infection on these cellular compartments [220]. Notably, a positive association between HIV-specific Tfh cells and class-switched memory B cells was observed implying that quality GCs activities may be critical for class-switching. This would enable antibodies to gain other Fc-mediated effector functions and enhanced Fab functions.

Table 3.5: Association of T and B cell subsets determined at 3 months PI.

	CD4+	CD45RA- CXCR5+ CD4+	PD-1+ CXCR3- CXCR5+ CD4+	ICOS+ PD-1+ CXCR5+ CD4+	CD27+ CCR7+ CD4+	CD27- CCR7- CD4+	HIV-specific CD45RA- CXCR5+ CD4+
Mature B cells	**0.279 (0.048)**	-0.175 (0.219)	-0.176 (0.218)	0.008 (0.955)	0.024 (0.867)	-0.220 (0.120)	0.058 (0.685)
Class switched B cells	-0.196 (0.169)	**0.322 (0.021)**	0.01389 (0.923)	-0.112 (0.434)	0.043 (0.766)	0.037 (0.792)	-0.137 (0.336)
Atypical B cells	-0.053 (0.709)	-0.054 (0.705)	-0.046 (0.746)	-0.118 (0.408)	**-0.280 (0.046)**	0.175 (0.220)	0.019 (0.895)
Naïve B cells	**0.411 (0.003)**	-0.165 (0.245)	-0.035 (0.810)	0.001 (0.992)	-0.124 (0.386)	0.010 (0.942)	-0.046 (0.749)
Activated B cells	**-0.389 (0.004)**	0.148 (0.301)	0.094 (0.514)	0.068 (0.635)	0.130 (0.360)	-0.003 (0.983)	0.158 (0.267)
Resting B cells	**-0.069 (0.626)**	0.096 (0.503)	-0.005 (0.973)	0.178 (0.210)	**0.359 (0.009)**	-0.206 (0.148)	-0.121 (0.393)
HIV-specific B cells	-0.048 (0.746)	0.178 (0.211)	0.008 (0.958)	0.275 (0.051)	0.070 (0.625)	-0.002 (0.985)	0.005 (0.972)

General and HIV-specific T and B cell subsets determined at 3 months PI were associated. In each cell, the Spearman's rho value and the associated p-value are shown. Bold blue indicates significant associations. Test; Spearman's non-parametric correlation, PI; post infection. $p<0.05$ was considered statistically significant.

3.4: Discussion

The integrity of the B and T cell compartments in HIV has been explored in-depth, in a bid of understanding how quality anti-HIV antibodies are generated. Despite many studies done in both macaques and human participant samples [86, 96, 99, 100, 111, 117, 126, 129, 221-223], we are still not certain how quality anti-HIV antibodies are generated. This study focused on understanding the early T and B cells interactions in HIV disease. T follicular helper cells are the CD4+ T cell subset that offers help to B cells in the B cell follicles [75], and the recent discovery of memory blood circulating Tfh cells [98] has enabled this study to characterize and evaluate the

interaction of these two subsets in HIV infection using PBMCs. Additionally, this study evaluated the association between the described early HIV-specific and general T and B cell subsets with downstream HIV-specific antibody levels and functions in chronic infection, as will be discussed later in chapters 4 and 5.

In the current study and also consistent with existing literature [86], an expansion of memory Tfh cells despite the depletion of the general CD4+ T cell frequencies was observed. The expansion may probably be attributed to the constant antigenic stimulation in HIV infected individuals or the relative decrease in the frequencies of other circulating CD4 T subsets [220]. Depletion of CD4+ effector memory T cell compartment was also observed, and this is supported by reports where effector memory T cell subsets were shown to be more likely to undergo apoptosis due to direct and indirect viral effects [220]. Upregulation of PD-1 on CD4+ T cells was also observed in HIV infected individuals. The higher signalling of PD-1 on CD4+ T cells has been documented [224] and is attributed to the direct HIV viral effects that may lead to cell activation or exhaustion [86]. Indeed, it has been previously suggested that increased expression of PD-1 in HIV infected individuals may limit quality antibody generation in HIV infection. Cubas and colleagues showed that PD-1/PD-L1 interaction leads to reduced cellular proliferation and inadequate IL-21 secretion and that the blocking of PD-1 signalling enhanced HIV-specific antibody generation in vitro [86]. Higher PD-1 signalling may, in a way present one of the earliest Tfh-cells' dysfunction, but is also crucial for Tfh function, as evident in PD-1 deficient mice that had poor Tfh function [89].

Despite these alterations in CD4+ T cells, this study has provided evidence that HIV-specific Tfh cells exist and circulate, albeit at low frequencies. Perhaps, the low frequencies of HIV-specific

Tfh cells in blood may be due to their sequestration in lymphoid tissue B cell follicles during an active HIV infection as proposed elsewhere [225]. Their identification through the co-expression of T cell activation markers (CD25 and OX40) is more sensitive and specific, as previously illustrated [161, 162]. However, these HIV-specific Tfh cells did not negatively correlate with HIV viral loads or positively with CD4 counts, and this may be due to, at least in part, the low frequencies of HIV-specific Tfh cells that were detected. However, there were significant associations between HIV-specific CD45RA- CXCR5+ CD4+ T cells and ICOS+ PD-1+ CXCR5+ CD4+ T cells, and ICOS+ PD-1+ CXCR5+ CD4+ T cells and PD-1+ CXCR3- CXCR5+ CD4+ T cells, suggesting a coordinated GCs activity. The GCs activity has previously been reported to be mechanistically linked [134].

Worth noting is that the frequency of CMV-specific memory Tfh cells in HIV-naïve and infected individuals were similar, suggesting that one does not lose CMV-specific memory Tfh cells after exposure to HIV. This may suggest that vaccinating HIV infected individuals against some diseases may still bear favourable vaccine efficacies. Activation due to SEB yielded similar cellular responses in both HIV infected and naïve individuals suggesting that CD4+ T cells respond similarly to a superantigen, regardless of HIV status, unlike in previous findings [226, 227]. However, despite the constant activation by HIV antigens in HIV infected individuals, there was no significant difference in the background immune activation between HIV infected and uninfected individuals, as determined by the unstimulated control. Perhaps a reflection of the still relatively stable immunological environment in the HIV infected individuals as this analysis were done 3 months post infection, prior to immunological deterioration.

Similar perturbations due to HIV infection were reported in the B cell compartment with an expansion of the TLM and activated memory B cell subsets being observed in HIV infected individuals, as has previously been reported [101]. Notably, TLM B cell subset had a positive correlation with HIV viral load measurements at 48 months PI, suggesting that high HIV viremia drives extensive immune activation and exhaustion that may lead to an expansion of the TLM subset over time [218]. Alternatively, TLM B cells may be involved in the spread of HIV to CD4+ T cells. B cells have been described to bind to HIV virions via the CD21 receptor that is expressed on mature B cells and later spread these virions to susceptible cells [21]. Notably, while it is generally agreed that HIV cannot productively infect and replicate in B cells, CD21 receptor interacts with HIV virions bound to complement proteins and hence facilitating HIV transmission from B cells to activated CXCR5+ CD4+ T cells [21, 228]. Since TLM B cells may either be $CD10^{low}/CD10^{-}$ [119], there is a possibility of their involvement in HIV transmission to susceptible CD4+ T cells.

HIV-specific memory B cells were detected at low frequencies in HIV infected individuals as has been previously observed, suggesting a depletion in the resting memory compartment as a result of HIV infection. Several studies have shown that HIV infected individuals have lower antibody levels against HIV-unrelated vaccines and pathogens due to the high HIV viremia and low CD4+ T cell counts [229-231]. In this study, cellular responses to CMV suggested that despite HIV infection, robust cellular responses against CMV were maintained, implying that revaccination against other pathogens would be beneficial. In the advent of test and treat in HIV infections as recommended by WHO [54], it would be important to explore the antibody responses of HIV-unrelated vaccines in individuals on ART. This would create a clearer understanding of vaccine-antibody responses to different pathogens in HIV infected individuals who are on ART.

Vaccination of HIV infected children who are on ART has been shown to maintain vaccine responses [232]. Similar to the HIV infected children on ART, it has been shown that the prior exposure to HIV in HIV-exposed uninfected infants did not affect serological responses in the first 2 years of life [233].

To gain insights into the interaction between T cell and B cell subsets in HIV, the frequencies of both subsets were associated. The frequencies of CD4+ T cells were positively associated with mature B cells, naïve B cells and AM B cells. The association with AM B cells may imply the importance of CD4+ T cells help to B cells in the GCs. As suggested elsewhere, it is not clear if the high concentration of HIV antigens in the draining lymph nodes drives the activation of RM B cells [217]. However, since the present study reveals a negative association between CD4+ T cells and RM B cells, and ART has been shown to block changes in the B cell compartment [217], it is plausible that HIV viremia is a significant driver of the perturbations in the B cell compartment.

Further analysis revealed that memory Tfh cells positively correlated with class-switched B cells. This association suggests that memory Tfh cells are critical for B cell class-switching in the GCs, and is consistent with the literature [105, 210]. Indeed, other general Tfh cell subsets in the peripheral circulation such as CXCR3+ CXCR5+ and PD-1+ CXCR3- CXCR5+ have been shown to offer better B cell help in T and B cell co-cultures [98, 234]. The CD27+ CCR7+ CD4+ T cells, which are memory T cells, were inversely associated with TLM B cells, probably due to the effect of HIV on the T and B cell compartments as earlier discussed. Since HIV viremia favours an increase of central memory CD4+ T cells [235], biologically, the HIV viremia would also deplete RM B cells.

Nevertheless, key associations reported here create a basic understanding of cellular biology in HIV disease, which is vital in understanding which cellular subsets may be correlates of quality antibodies in HIV. For instance, mature B cells and ICOS+ PD-1+ CXCR5+ CD4+ T cells negatively correlated with HIV viral load, suggesting that they may be key in controlling HIV or may represent markers of disease progression. Similar to these findings, the frequencies of memory B cells were reported to positively correlate with CD4+ T cell counts and thus represent a marker of disease progression [229]. Thus, future studies should address approaches that favour the preservation of functional subsets, as that may be key in the effective control of HIV infection. Such an approach may either include the potential use of appropriate adjuvants that will favour the generation and maintenance of relevant subsets or the exclusion of proteins in potential immunogens that would trigger unforeseen and unfavourable immunological consequences that lead to perturbations in these functional subsets.

This data suggests that the frequencies of HIV-specific T- and B cell subsets may describe HIV disease progression rates and that high HIV viremia may be required for stimulating and sustaining HIV-specific B cell responses. In summary, the results in this chapter showed that:

- HIV infection depletes RM B cells and drives the expansion of TLM and AM B cells.
- HIV-specific B cells circulate at low frequencies and this may be due to the direct and indirect effects on the B cell compartment by HIV infection.
- HIV depletes CD4+ T cells and CD4+ effector memory T cells and expands CD4+ central memory T cells.
- HIV-specific Tfh cells circulate at low frequencies in blood, perhaps as a result of the sequestration of HIV-specific Tfh cells in lymphoid tissue B cell follicles during an active HIV infection.

While these findings should be treated with caution due to the low frequencies of HIV-specific T and B cells detected, this study has described early HIV-specific T- and B cell subsets which may help to understand HIV-specific responses. Future larger studies could confirm these findings and provide additional insights on HIV-specific responses.

Chapter 4 Plasma levels of CXCL13, BAFF and HIV-specific antibody isotypes and subclasses in the course of HIV infection

4.1: Literature review

It is widely accepted that the development of a successful anti-human immunodeficiency virus (HIV) vaccine will likely require the elicitation of antibodies against HIV, and more desirably, antibodies with potent neutralising abilities [110, 236-239]. However, Fc-mediated antibody functions may potentially play a role [240-244]. Apart from antibody functions (quality), antibody quantities may also be critical. For instance, anti-HIV antibody levels have been linked with quality Fab-mediated antibody responses in a large longitudinal Sub-Saharan HIV primary infection cohort [110]. Moreover, mice vaccinated with the immune complexes from HIV neutralisers displayed higher levels of overall antibody titres than those vaccinated with immune complexes from HIV non-neutralisers [245]. This may suggest an association between antibody quantities and qualities in HIV infection. Since all these critical immunological processes occur in the germinal centres (GCs), GCs activities may, therefore, determine the quality and quantity of antibody responses, with an increased GCs activity favouring both [246], since there is a mechanistic link between the Fc and Fab-mediated antibody responses [134].

The chemokine CXCL13 and the B cell activating factor (BAFF) are critical in GCs formation. The chemokine CXCL13 is involved in organising and maintaining the B cell follicles in GCs that may lead to the generation of broadly neutralising antibodies (bnAbs), as reported in HIV infection [162]. Through its receptor CXCR5, CXCL13 is responsible for the migration of B and T-follicular helper cells (Tfh cells) to the B cell follicle [212, 247]. BAFF is also present in the lymph node and is key in B cell-related immune responses. BAFF plays a vital role in B cell homeostasis and has been shown to influence the survival and class switching of B cells in general [97, 248]. Also, BAFF has been shown to support the survival of autoreactive B cells that are capable of generating

cross-neutralising anti-HIV antibodies [249-251]. Despite both CXCL13 and BAFF cytokines acting locally in the lymph node tissues, both are readily quantifiable in plasma. CXCL13 has previously been associated with the level of GCs activity [96] and the development of anti-HIV antibodies with cross-reactive activities [101].

In addition to supporting T and B cell interaction that results into B cell differentiation, antibody production and affinity maturation, the GCs reactions also support class switching from immunoglobulin (Ig) M and IgD expression to IgG, IgE and IgA expression. Furthermore, depending on the cytokine environment in the GCs, IgG could class switch to any of the subclasses IgG_1, IgG_2, IgG_3 or IgG_4 [174] with interleukin (IL) -4, IL-5, interferon-gamma (IFN-γ) and transforming growth factor-beta *(TGF-β)* being the cytokines mainly involved in antibody class switching. Interleukin 4 induces switching to IgG_1 and IgE while inhibiting switching to IgG_3. The *TGF-β* similarly inhibits switching to IgG_3 and favours switching to IgG_2 and IgA. The cytokine IFN-γ cytokine induces switching to IgG_3 and IgG_2 while inhibiting switching to IgG_1 and IgE. Interleukin-5 only favours switching to IgA and does not affect the other isotypes [174].

Antibody class switching confers new effector functions (Fc-mediated) to the antibody without changing its specificity. In HIV infection, anti-Env antibodies are predominantly IgG and IgG_1 [252, 253], with the latter associated with antibody-dependent cellular cytotoxicity (ADCC) of HIV-infected cells [254]. Despite anti-ENV IgG_2 being detected throughout HIV infection [144, 255], the levels are lower relative to other subclasses [252, 256, 257]. Anti-Env IgG_3 is the second most predominant IgG subclass after IgG_1 [258] and has been shown to have superior in-vitro neutralising ability probably due to the greater flexibility of the immunoglobulin hinge region [259].

Unlike the other subclasses, HIV-specific IgG_4 is mostly detectable in chronic HIV infection [254, 260], suggesting that chronic antigenic stimulation may be critical for their switching. Indeed, HIV-specific IgG_4 responses have been reported in individuals in regions where there are constant multiple exposures to viral, bacterial, and parasitic antigens [261], further highlighting the role of chronic antigen stimulation of IgG_4 predominance. Other subtypes such as IgA antibodies also play a crucial role in protection against HIV. For instance, HIV-specific IgA is protective against HIV infection [262]. HIV-specific neutralising IgA in HIV uninfected sex workers was associated with protection from HIV acquisition [263, 264]. Despite these data, it is not clear if HIV mucosal vaccination will be protective or not. However, these anti-HIV responses of multiple isotypes and subclasses have been reported to be similar in Env, Gag, and Pol proteins [265]. In disease, the different antibody subclasses and isotypes mediate varying effector functions with different efficacies as summarized below in Table 4.1.

Table 4.1: The effector functions of different immunoglobulin isotypes and subclasses.

Fab/Fc functionality	**IgD**	**IgM**	**IgG_1**	**IgG_2**	**IgG_3**	**IgG_4**	**IgE**	**IgA**
Opsonization (Monocyte and neutrophil killing)	-	+	+++	++	++	++	-	++
Natural killer cell killing	-	-	++	-	++	-		-
Mast cell sensitization	-	-	+	-	+	-	+++	-
Complement activation	-	+++	++	+	+++	-	-	+
Neutralisation ability	-	+	++	++	++	++	-	++

(+++) represents major effector function, (++) lesser effector function, and (+) minor effector function. (From Janeways Immunobiology [174]).

In this chapter, I determine the plasma quantities of HIV-specific total IgG, IgG_1, IgG_2, IgG_3, IgG_4, IgM, IgA_1 and IgA_2 and the plasma levels of the B cell cytokines, CXCL13 and BAFF. I further establish associations of the soluble factors CXCL13 and BAFF and resulting antibody levels with HIV disease progression (HIV viral load measurements and CD4+ T cell counts).

4.2: Objective

To determine HIV-specific antibody levels, isotypes and subclasses during HIV infection and the plasma levels of BAFF and CXCL13 cytokines, as a proxy of germinal centre activities, before and during HIV infection.

4.3: Results

4.3.1: HIV-specific antibody isotypes and subtypes levels

HIV-specific antibody isotypes and subtypes levels were determined by an in-house enzyme-linked immunosorbent assay (ELISA) and a Luminex isotype assay as described in Chapter 2, subsections 2.8 and 2.9.

By ELISA, HIV-specific IgG, IgG_1, IgG_2, IgG_3 and IgA were detectable at all time points assessed (3-, 12- and 48- months PI). HIV-specific IgG, IgG_1 and IgA levels significantly increased as the disease progressed, $p<0.0001$, $p<0.0001$ and $p=0.002$ respectively. The levels of HIV-specific IgG_2 and IgG_3 increased up to 12 months PI, before slightly decreasing at 48 months PI. HIV-specific IgM was only detected at 3 months PI. HIV-specific IgG_4 levels were below the limit of detection for this assay and not detected at any of the time points (Figure 4.1).

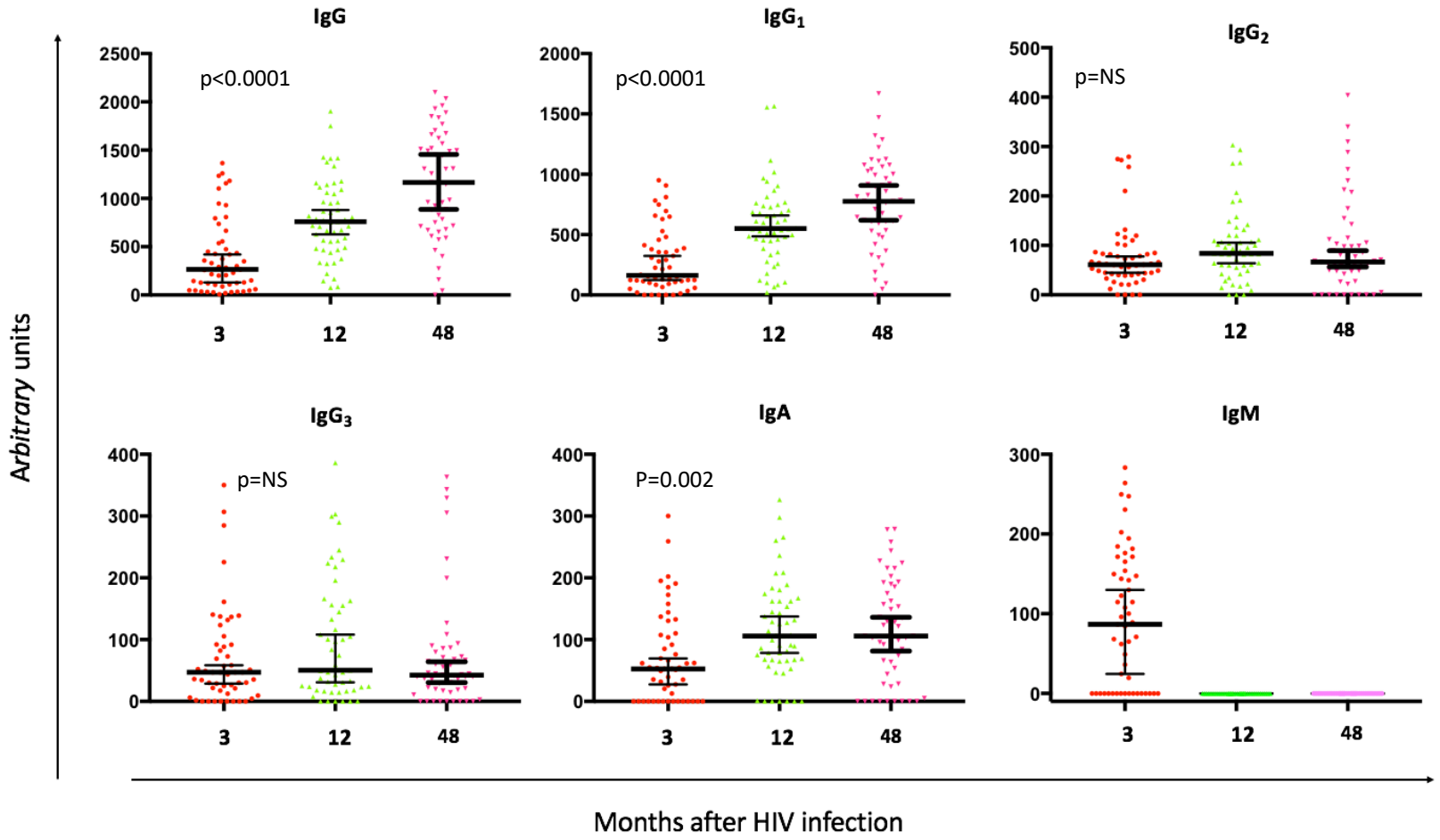


Figure 4:1: The kinetics of HIV-specific antibody isotypes and subclasses levels after HIV infection as detected by ELISA. The median concentration for each sub-group and IQR value is shown. The x-axis represents time in months after HIV infection while the y-axis represents the arbitrary units of concentration of each sub-group. Test, Kruskal-Wallis. Ns; not significant.

In order to test a broader range of antigens, a multiplex microsphere bead assay was used. A similar pattern of HIV-specific antibody subclasses and isotypes levels was observed. Similar to the ELISA data, higher levels of IgG and IgG_1 were observed. However, unlike the ELISA data, HIV-specific IgM and IgG_4 levels were detected at all time points (3-, 12- and 48 months PI) (Figure 4.2).

Since a wide range of HIV antigens from clades A, B and C were used in the Luminex assay, the presence of cross-reactivity antibodies was determined. Cross-reactive antibodies were detected at different levels for each antigen tested. The 94UG, although a clade A protein, representing the clade prevalent in this region, consistently showed lower responses across all time points. The flu and tetanus antigens were used as controls based on prior exposure and vaccination respectively. As would be expected, high responses to tetanus antigens were observed while those to the flu antigens were moderate across all the time points. High levels of tetanus specific IgM levels were observed throughout the follow up period (Appendix 7). Since IgM is the first antibody to be generated in the humoral immune response [266], we would have expected that these levels wane during follow up. It is not exactly clear why these levels were maintained. It is however possible that by stander activation in these highly immune activated individuals may be contributing to these levels as reported elsewhere [267]. It is also possible that some of the participants could have had booster doses or natural exposure in adulthood. While only data of IgG and IgG_1 antibodies levels to all antigens are presented in Figure 4.2, data on all antibody isotypes and subclasses to all antigens are presented in Appendix 6.

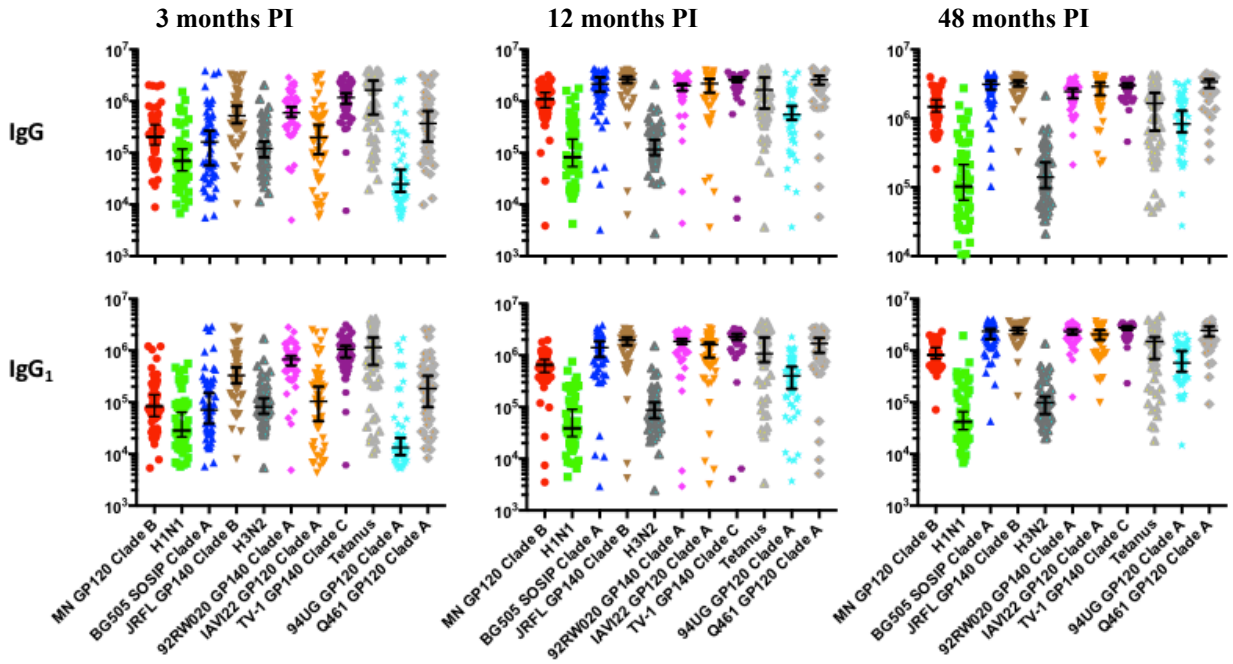


Figure 4:2: The dynamics of HIV-specific IgG and IgG_1 levels after HIV infection as detected by the Luminex assay. Eight HIV antigens spanning across clades A, B and C were used to detect HIV-specific responses while flu and tetanus antigens were used as controls. The median concentration for each sub-group and IQR values are shown. The y-axis represents mean fluorescence intensity (MFI) values; the x-axis represents different antigens used. PI represents post-HIV infection. Test, Kruskal-Wallis.

The different HIV antigens used showed similar patterns of antibody levels across time, and the data from the BG505 clade A antigen, representative of the prevailing clade in the region, was therefore used for downstream analysis and is presented in Figure 4.3. BG505 SOSIP Clade A gp120 specific antibody levels increased over time as HIV disease progressed (Figure 4.3). Higher levels of HIV-specific IgG_1 and IgG_3 were observed compared to the levels IgG_2 and IgG_4 responses, which remained low. Interestingly, while all isotypes and subclasses significantly increased over time, there was no significant increase in IgG_3 ($p=0.189$). In summary, HIV infection drives higher HIV-specific antibody levels as the disease progresses.

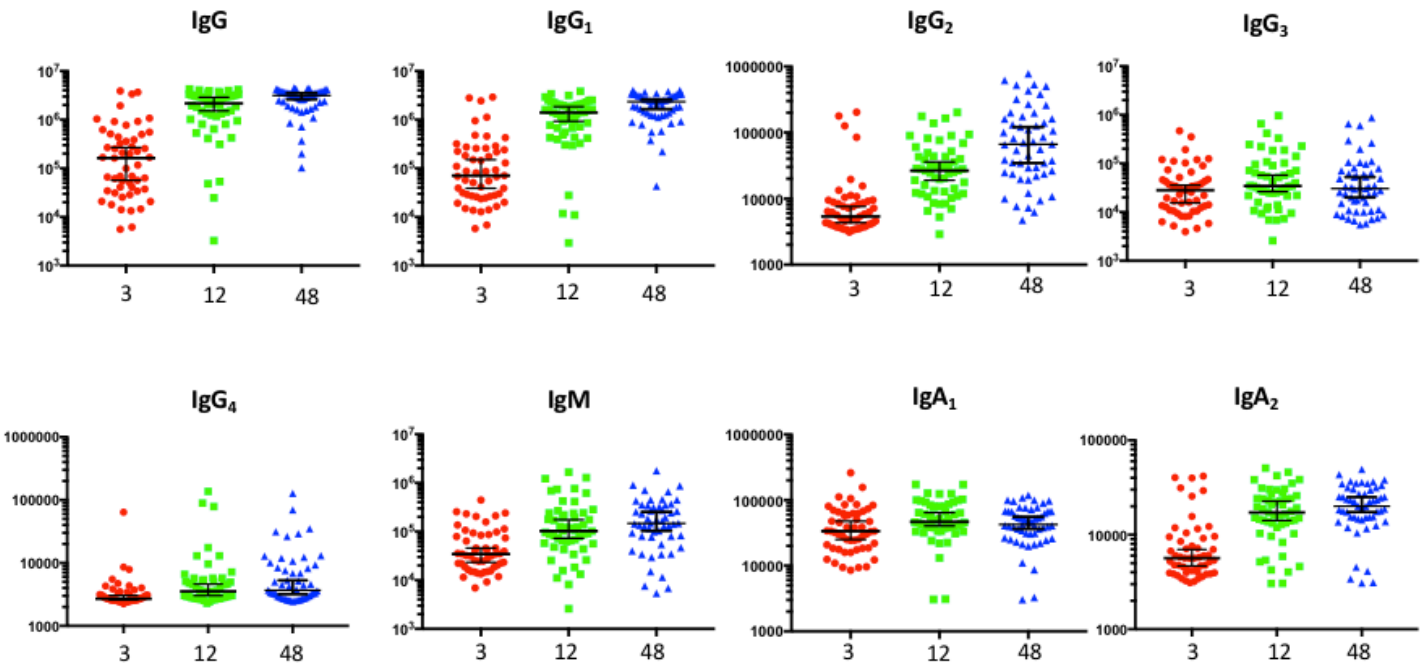


Figure 4:3: The dynamics of BG505 clade A HIV-specific antibody isotypes and subclasses levels after HIV infection as detected by the Luminex assay. The median concentration for each antibody isotypes and subclasses and IQR values are shown. The y-axis represents mean fluorescence intensity (MFI) values; the x-axis represents the different time points of sampling, 3-, 12- and 48-months PI. Test, Kruskal-Wallis.

4.3.2: The kinetics of CXCL13 and BAFF before and during HIV infection

To check if intrinsic GCs activity influences HIV disease outcomes, BAFF and CXCL13 levels were determined before HIV infection as a proxy for GCs activity, and longitudinally after HIV infection to check for the effect on the B cell cytokines after to HIV infection. A drop in BAFF

and CXCL13 was observed after HIV infection compared to levels in pre-infection samples; 1556 versus 1018, p<0.0001 and 170.4 versus 131.7, p=0.034 respectively (Figure 4.4).

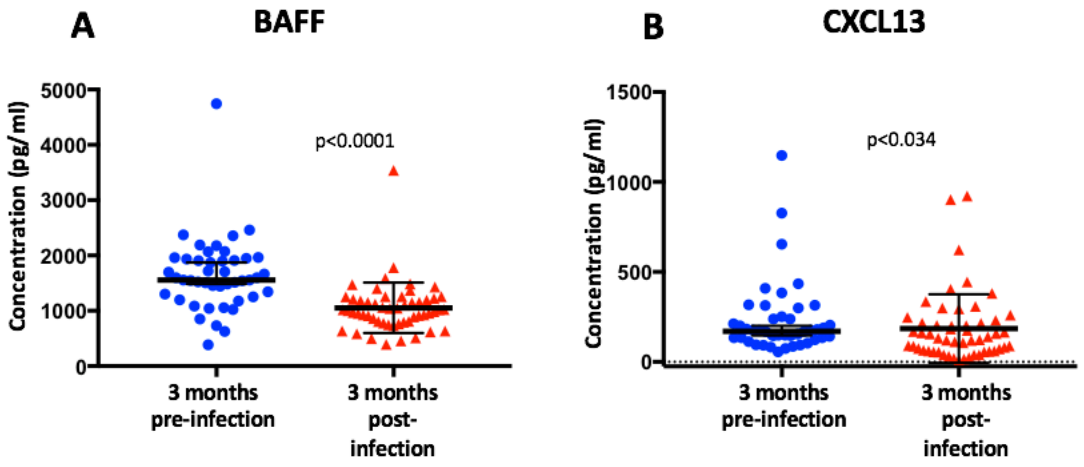


Figure 4:4: Dynamics of plasma BAFF and CXCL13 levels following HIV subtype A infection. Panels **(A, B)** show kinetics of BAFF and CXCL13 plasma levels determined by ELISA on HIV pre-infection plasma samples (3 months before infection) and on plasma samples at 3 months PI. Horizontal lines represent median values and the associated IQR values. p-values were calculated by Mann-Whitney test. p-values <0.05 was considered significant.

The median BAFF concentration at 3 months PI was 1018 pg/ml (IQR, 800.4-1214), at 12 months PI, 759.7 pg/ml (759.7 (464.8-1337) and 48 months PI, 1065 pg/ml (IQR, 503.3-1627) (Figure 4.4). The median CXCL13 concentration at 3 months pre-infection was 170.4 pg/ml (IQR, 134.1-245.3), at 3 months PI, 131.7 pg/ml (IQR, 60.63-223.6), at 12 months PI, 146.9 pg/ml (83.67-294) and 48 months PI, 110.2 pg/ml (IQR, 65.9-19).

After HIV infection, BAFF levels transiently decreased earlier in HIV infection but returned to similar levels observed prior to infection by 48 months PI. CXCL13 plasma levels decreased after HIV infection and remained relatively low up to towards 48 months PI. It appears that HIV

infection drives a transient decline in the levels of the B cell cytokines, BAFF and CXCL13, soon after infection (Figure 4.5).

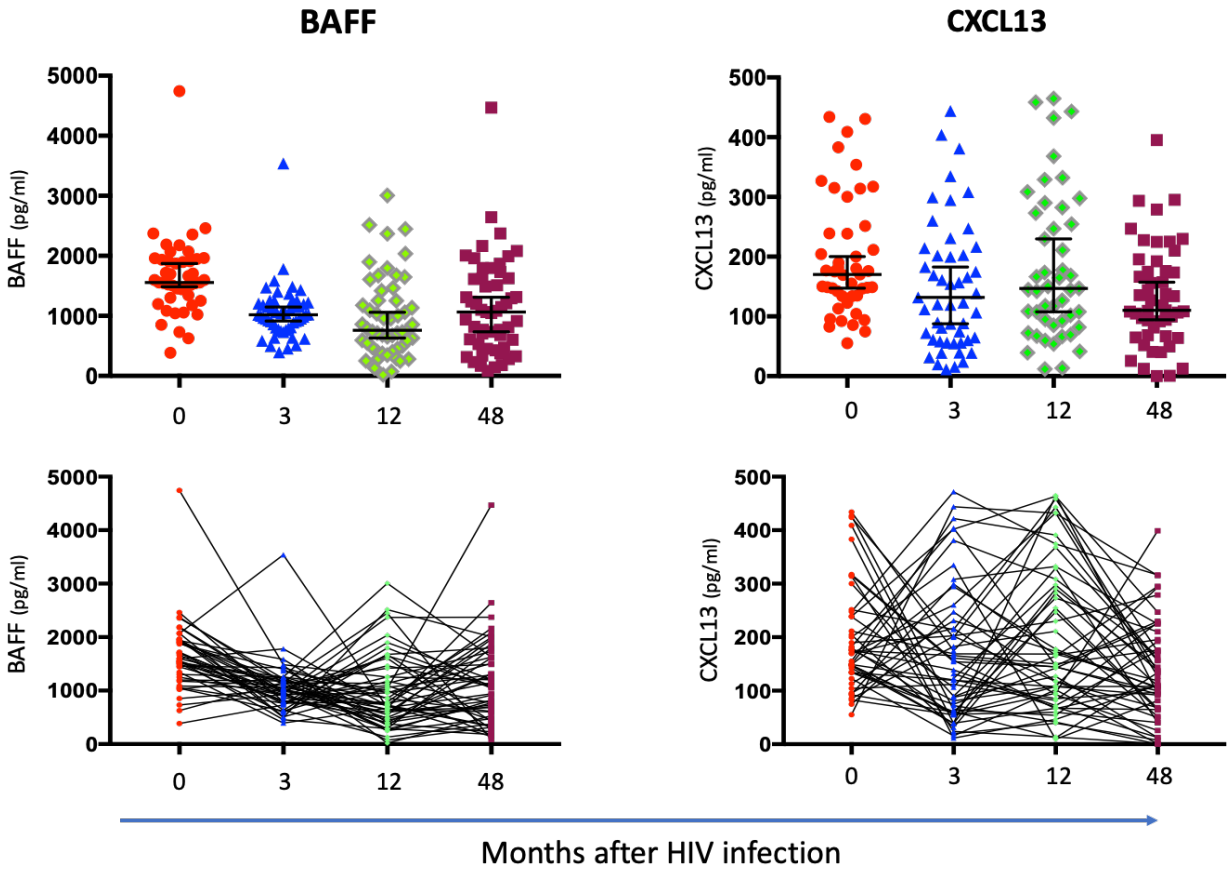


Figure 4:5: The dynamics of BAFF and CXCL13 plasma levels 3 months before HIV infection (0) and 3-, 12- and 48 months after HIV infection. Panels on the right and left show the kinetics of median levels of BAFF and CXCL13, respectively, that were longitudinally obtained from HIV pre-infection (0) to 48 months post-HIV infection. There were no significant differences between the time points. (Kruskal-Wallis test).

4.3.3: Association between BAFF and CXCL13 cytokines levels

As BAFF and CXCL13 cytokines are essential in driving GCs activities, their association during HIV infection was done. At 3 months PI, BAFF and CXCL13 levels were positively associated rho=0.31, p=0.024, but this association was lost as the disease progressed at 12- and 48- months PI (Figure 4.6). Notably, unlike BAFF levels, individuals who generated high levels of CXCL13 earlier in HIV infection maintained high levels at later stages of the infection and vice versa,

implying that HIV infected individuals who develop quality responses earlier in infection are likely to consistently make such responses in chronic infection (Appendix 7).

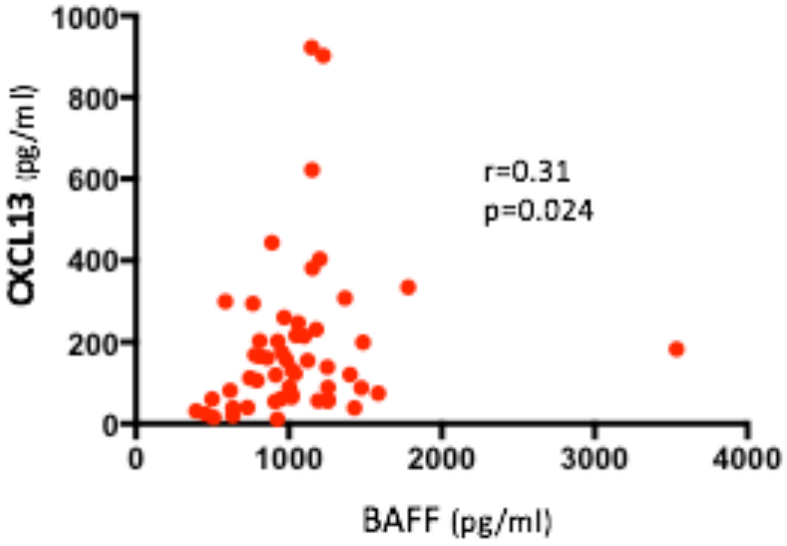


Figure 4:6: Correlation between BAFF and CXCL13 at 3 months PI. The x-axis represents BAFF concentration in pg/ml while the y-axis represents CXCL13 concentration in pg/ml. Statistical test: Spearman's correlations. P-values <0.05 was considered significant.

4.3.4: Correlation between the levels of B cell cytokines and HIV-specific antibody levels

In order to assess the association between HIV-specific antibody levels and B cell cytokines, Spearman's correlation was done. The levels of the cytokine BAFF had no significant association with any of the HIV-specific antibody isotype or subclass. However, CXCL13 levels positively associated with most antibody subclasses and isotypes at 3 months PI. This positive association was maintained as HIV disease progressed to 48 months PI, at least for IgA1, IgG_1, IgG, and IgG_3 (Table 4.2).

Table 4.2: Correlation between HIV-specific antibody isotypes and subclasses levels at 3 months PI with CXCL13 levels at 3-,12- and 48 months PI.

	IgA1	**IgA_2**	**IgG_1**	**IgG**	**IgM**	**IgG_3**
CXCL13 (3 months PI)	0.33 (0.01)	0.42 (0.002)	0.56 (<0.0001)	0.54 (<0.0001)	0.484 (0.0002)	0.49 (0.0002)
CXCL13 (12 months PI)	0.37 (0.007)		0.37 (0.007)	0.56 (<0.0001)		0.28 (0.036)
CXCL13 (48 months PI)	0.41 (0.002)		0.44 (0.001)	0.434 (0.001)		0.27 (0.04)

The top row represents HIV-specific antibody isotypes and subclasses at 3 months PI while the left column represents CXCL13 levels at 3-, 12- and 48 months PI. Spearman's rank correlation was used, and all p-values are shown in brackets in the cells. PI- post-HIV infection. Only significant associations are shown.

Similarly, only CXCL13 had significant positive associations with HIV-specific antibodies at 12 months PI. The HIV-specific antibody subclasses and isotypes that had significant associations included IgA_2, IgG_1, IgG, IgM, IgG_2 and IgG_3 (Table 4.3). BAFF levels measured at different time points had no significant association with any of the HIV-specific isotype or subclass levels.

Table 4.3: Correlation between HIV-specific antibody isotypes and subclasses levels at 12 months PI with CXCL13 levels at 3-, 12- and 48 months PI.

	IgA_2	**IgG_1**	**IgG**	**IgM**	**IgG_2**	**IgG_3**
CXCL13 (3 months PI)	0.34 (0.012)	0.39 (0.005)	0.38 (0.004)	0.52 (<0.0001)	0.37 (0.006)	0.49 (0.0002)
CXCL13 (12 months PI)		0.32 (0.01)	0.34 (0.012)	0.53 (<0.0001)	0.28 (0.04)	0.28 (0.036)
CXCL13 (48 months PI)				0.33 (0.014)		0.27 (0.04)

The top row represents HIV-specific antibody isotypes and subclasses at 12 months PI while the left column represents CXCL13 levels at 3-, 12- and 48 months PI. Spearman's rank correlation was used, and all p-values are shown in brackets in the cells. PI- post-HIV infection. Only significant associations are shown.

At 48 months PI, both CXCL13 and BAFF levels at 3 months PI had significant positive associations with some of the HIV-specific antibody subclasses and isotypes (Table 4.4). Specifically, CXCL13 levels at 3, 12 and 48-months PI had significant associations with either

IgA2, IgG_1, IgG or IgG_2 at 48 months PI. Unlike CXCL13 plasma levels, BAFF plasma levels at 3 months PI only had significant associations with IgA2 and IgG levels at 3 and 12-months PI respectively.

Table 4.4: Correlation between HIV-specific antibody isotypes and subclasses levels at 48 months PI with CXCL13 levels at 3- ,12- and 48 months PI.

	IgA2	**IgG_1**	**IgG**	**IgG_2**
CXCL13 (3 months PI)	0.28 (0.004)	0.33 (0.001)	0.37 (0.006)	0.35 (0.01)
CXCL13 (12 months PI)		0.42 (0.014)	0.34 (0.012)	
CXCL13 (48 months PI)	0.29 (0.038)		0.498 (0.0001)	0.41 (0.003)
BAFF (3 months PI)	0.31 (0.004)			
BAFF (12 months PI)			0.28 (0.005)	

The top row represents HIV-specific antibody isotypes and subclasses while the left column represents CXCL13 and BAFF levels at 3-, 12- and 48 months PI. Spearman's rank correlation was used, and all p-values are shown in brackets in the cells. PI- post-HIV infection. Only significant associations are shown.

4.3.5: Correlation between HIV-specific antibody levels with the markers of HIV disease progression

HIV disease progression was determined based on HIV viral load measurements and CD4+ T cell counts change over time. When associating HIV-specific antibodies with markers of HIV disease progression, a positive correlation was observed; IgA_1 versus CD4+ T cell counts at 3 months PI (rho=0.295, p=0.03), IgM versus CD4+ T cell counts at 48 months PI (rho=0.295, p=0.03), IgG_1 versus viral load measurements at 3 months PI (rho=0.38, p=0.05) and versus viral load measurements at 48 months PI (rho=0.28, p=0.04). Similar associations were seen for IgG and IgG_2 with viral load measurements at 3 months PI, (rho=0.29, p=0.03) and (rho=0.36, p=0.026), respectively. There was no correlation between CD4+ T cell counts and viral load measurements at 12 months PI (Table 4.5).

Table 4.5: Correlation between HIV-specific antibody isotypes and subclasses levels at 3 months PI with CD4+ T cell counts and HIV viral load measurements at 3-, 12- and 48 months PI.

	IgA_1	**IgM**	**IgG_1**	**IgG**	**IgG_2**
CD4 counts (3 months PI)	0.295 (0.03)				
CD4 counts (12 months PI)					
CD4 counts (48 months PI)		0.31 (0.02)			
Viral load (3 months PI)			0.38 (0.005)	0.29 (0.033)	0.36 (0.026)
Viral load (12 months PI)					
Viral load (48 months PI)			0.28 (0.04)		

The top row represents HIV-specific antibody isotypes and subclasses, while the left column represents markers of HIV disease progression at 3-, 12- and 48 months PI. Spearman's rank correlation was used, and all p-values are shown in brackets in the cells. PI- post-HIV infection. Only significant associations are shown.

On investigating the association of antibodies to disease progression at 12 months PI, IgA_1 was positively associated with absolute CD4+ T cell counts determined at 3-, 12- and 48-months PI: (rho=0.35, p=0.01) at 3 months PI, (rho=0.37, p=0.0065) at 12 months PI and (rho=0.41, p=0.0023) at 48 months PI. Additionally, IgG_1, IgG and IgG_2 positively correlated with HIV viral load measurements at 3 months, (rho=0.27, p=0.04), (rho=0.3, p=0.04) and (rho=0.36, p=0.008) respectively. These positive associations were also observed with viral load measurements at 12 months PI for IgG_1, IgG and IgG_2 with (rho=0.4, p=0.0032), (rho=0.38, p=0.005) and (rho=0.38, p=0.005) respectively (Table 4.6). However, no significant associations were observed between HIV-specific antibody levels at 48 months PI with the B cell cytokines levels, HIV viral load measurements and CD4+ T cell counts at different time points.

Table 4.6: Correlation between HIV-specific antibody isotypes and subclasses levels at 12 months PI with CD4+ T cell counts and HIV viral load measurements at 3-, 12- and 48 months PI.

	IgA1	**IgG_1**	**IgG**	**IgG_2**
CD4 counts (3 months PI)	0.35 (0.01)			
CD4 counts (12 months PI)	0.37 (0.007)			
CD4 counts (48 months PI)	0..41 (0.002)			
Viral load (3 months PI)		0.27 (0.04)	0.3 (0.04)	0.36 (0.008)
Viral load (12 months PI)		0.4 (0.003)	0.38 (0.005)	0.38 (0.005)
Viral load (48 months PI)				

The top row represents HIV-specific antibody isotypes and subclasses while, the left column represents markers of HIV disease progression at 3-, 12- and 48 months PI. Spearman's rank correlation was used, and all p-values are shown in brackets in the cells. Only significant associations are shown. P-values <0.05 were considered significant.

4.3.6: Correlation between the levels of B cell cytokines with the markers of HIV disease progression

On assessing associations between the B cell cytokines with the markers of HIV disease progression (HIV viral load measurements and CD4+ T cell counts), there was no significant correlation of BAFF levels with either of the markers of HIV disease progression. However, CXCL13 levels at 3 months PI positively correlated with viral load measurements at 3 months PI (Spearman rho=0.49, p=0.0002) and negatively correlated with CD4+ T cell counts at 3 months PI (rho=-0.36, p=0.00077). This may suggest that HIV viremia drives quality GCs activity, albeit, at the cost of depletion of CD4+ T cell counts. Similarly, it may point out to the migration of Tfh cells to the lymph nodes in the presence of CXCL13.

4.4: Discussion

This study investigated the levels and kinetics of BAFF, a cytokine important for B cell survival [97], and CXCL13, a chemokine responsible for B cell trafficking to GCs [268] before and during HIV infection. Unlike in previous studies where HIV has been associated with a cytokine storm after infection [29, 269-273] and an increase in BAFF and CXCL13 levels [217], the current data suggests otherwise. Perhaps this is likely due to the disparity in the sampling time points, where other studies used samples from earlier time points post HIV infection [273]. An initial observation of low BAFF and CXCL13 levels at 3 months post HIV infection prompted the measurement of these cytokines prior to infection and thus confirming the drop of these cytokines at 3 months PI. BAFF levels only significantly increased at 48 months PI, while the CXCL13 levels remained relatively low up to 48 months PI. It is not clear why the levels of CXCL13 decreased after HIV infection despite the effects of HIV immune activation and continuous viral replication within lymphoid tissues, which has been shown to elevate CXCL13 levels [217]. Perhaps the different findings may be due to the clade-specific effects, where Mabuka and others used a cohort of individuals infected with HIV clade C [217], unlike HIV clade A used in this study. HIV clade C has previously been associated with higher viral loads, lower CD4+ T cell counts and thus a faster HIV disease progression as compared to HIV clade A, perhaps due to increased replicative fitness [274], and this would drive robust immune activation and thus higher CXCL13 levels [217]. In addition to HIV clade-specific replicative fitness, CXCR4-tropism has been reported to influence HIV disease progression [275]. Also, it is possible that the cytokine storm may have occurred earlier than the 3 months PI, when the sampling was done.

Nevertheless, CXCL13 and BAFF levels had a positive association at 3 months PI suggesting a coordinated GCs activity since both cytokines act locally in the lymph nodes to enhance B- and T cell interactions and activations [276]. Additionally, individuals with high CXCL13 levels maintained similar responses and vice versa, which may imply that quality GC activities may be sustained throughout an infection. Moreover, this may also point out that early priming of GCs activities in disease may be necessary for quality GCs activities in chronic infection. This is further supported by the positive correlation between CXCL13 levels and viral load measurements, which emphasizes the need for high antigenic stimulation for quality GCs activity. The positive association between CXCL13 levels and viral load measurements has been reported elsewhere [277-279].

As expected, CXCL13 levels and CD4+ T cell counts were inversely associated possibly due to the migration of Tfh cells from the peripheral circulation to the lymph nodes in the presence of CXCL13. The weak association of BAFF and the markers of HIV disease progression has been described elsewhere, where, BAFF levels failed to show any association with viral load measurements [217]. However, HIV viremia is known to cause a depletion of B cells through inducing decreased responsiveness of B cells to BAFF, leading to apoptosis due to lack of pro-survival signals [280].

To determine the levels of HIV-specific antibody isotypes and subclasses, this study used a standard ELISA assay but later incorporated a multibead Luminex assay that permitted the measurement of a broader range of antigen responses. Results from the two assays were in agreement. However, the limit for detection was lower for the inhouse ELISA than for the Luminex assay. This is consistent with the findings from a Zaire Ebola study, where the Luminex-based

assay was shown to be as sensitive as ELISA and more specific and accurate in detecting the Zaire Ebola virus IgG in human plasma [281]. Indeed, the Luminex assay has successfully been used in different studies to assess antigen-specific antibody levels, cytokines, chemokines and heat shock proteins [134, 282-285]. Cross-reactivity to antigens from other HIV clades was observed due to antibodies in plasma targeting epitopes within conserved regions of the HIV ENV, as described elsewhere [286].

Despite the decreasing levels of the B cell cytokines reported, there was a significant increase in HIV-specific isotypes and subclasses antibody levels as HIV disease progressed. This may be attributed to the increase of HIV viremia, as reported in an HIV-elite and non-controller cohort [287]. Consistent with the literature, IgA isotype was found to circulate at low levels in plasma [288], with IgA_1 being more prevalent than IgA_2. This is consistent with the literature, where serum IgA_1 has been shown to circulate at higher levels than IgA_2 with a ratio of 9:1 [289]. While both IgA_1 and IgA_2 share significant sequence similarities, IgA_1 has an insertion of duplicated amino acids which creates an extension in its hinge region [290] and is more glycosylated [291].

Furthermore, and similar to other reports, IgG_1 was the most predominant subclass, IgG_3 the second most predominant while IgG_4 was the least dominant [256, 258, 292, 293]. However, despite the low levels of IgG_4 antibody responses, these responses significantly increased as HIV disease progressed. Similar findings have been shown in studies where HIV-specific IgG_4 antibody responses were readily available in chronically HIV infected patients [254, 260]. IgM was detectable, though in low levels in chronic HIV infection. This isotype IgM has previously been shown to be detected in chronic HIV disease, at least in low levels [112]. More likely, the significant increase in HIV-specific IgM as HIV disease progresses may be due to the constant

antigenic stimulation at the GCs that may lead to its generation, before class switching occurs [28, 287].

HIV-specific isotypes and subclasses antibody levels were associated with preservation of CD4+ T cell counts despite the positive correlation with HIV viral load measurements too. As previously discussed in a review [294], this may suggest that while the high viral load is required for higher antibody titres, preservation of CD4+ T cells (Tfh cells) is critical to achieving quality GCs activity that results in higher antibody titres.

As expected, CXCL13 levels were positively associated with HIV-specific isotypes and subclasses antibody levels. The cytokine CXCL13 organizes the B cell follicles of secondary lymphoid organs via recruiting antigen-specific B and T cells through the CXCR5 receptor [295]; thus, its association with antibody levels is expected. Similarly, BAFF was associated with higher levels of HIV-specific IgG. Since BAFF is essential for B cell survival factor [97], it is plausible that higher levels of BAFF would favour higher frequencies of B cells that would translate to higher levels of antigen-specific antibodies.

It appears that the overall increase in HIV-specific antibody isotypes and subclasses over time is due to the high viremia since all study participants were ART naïve, and ART is known to cause HIV viral suppression which leads to a progressive decline in antibody responses [287]. The implications of increasing titres of anti-HIV antibodies as HIV progresses differ. For instance, due to the narrowness of antibody responses against a quickly mutating HIV, antibody responses, despite their magnitude, may fail to control viremia leading to rapid viral escape [113, 114]. Additionally, while most anti-HIV antibody responses target the ENV on the viral surface [296], very few can recognise the conserved regions on the trimeric ENV [297, 298] and thus ineffective.

Paradoxically, HIV-specific antibodies may be beneficial to the virus through a process called complement-mediated antibody-dependent enhancement of viral infection, as shown in-vitro [299, 300]. The process involves the binding of an antibody to either gp-41 or gp-120 which initiates a complement cascade. Once the opsonized virions bind to CD21, the engagement of CD21 with CD4 leads to enhanced HIV replication [301]. However, higher HIV antibody titres have been have been associated with quality Fc polyfunctionality [134] in HIV infection and the development of bnAbs [134, 159], which may be beneficial for vaccine design.

In summary, these data show that:

- HIV infection drives higher HIV-specific antibody levels as the disease progresses.
- HIV infection drives a transient decline in the levels of the B cell cytokines, BAFF and CXCL13, soon after infection.
- BAFF and CXCL13 levels were positively associated earlier in early HIV infection and hence suggesting an immunological link in GCs activities.
- Unlike BAFF plasma levels, CXCL13 levels positively associated with most HIV-specific antibody subclasses and isotypes and thus implying that CXCL13 levels in early HIV infection may be a good predictor of HIV-specific antibody levels in chronic infection.
- Most of the HIV-specific antibody levels were positively associated with viral load measurements at different time points and thus suggesting a role of HIV viremia in driving higher levels of HIV-specific antibodies.
- The positive associations between plasma CXCL13 levels with HIV viral load measurements may imply that HIV viremia is necessary for quality GCs activity.

In this chapter, I have demonstrated that GCs activities, HIV viral load and HIV-specific antibody levels are positively associated, suggesting that they are immunologically linked. However, since B cells require help from CD4+ T cells, which are depleted in HIV infection, more studies will be needed to elucidate how these immunological activities could be exploited for the realization of an antibody-based HIV vaccine.

Chapter 5 Fc-mediated and Fab-mediated antibody functions in HIV infection

5.1: Literature review

Antibodies are the secreted form of the B cell receptor (BCR) and hence identical in structure except for a small portion of the C-terminus of the heavy-chain constant region. While the C-terminus in the BCR is a hydrophobic membrane-anchoring sequence, the C-terminus of the antibody is a hydrophilic sequence that allows its secretion [302]. Typically, antibody molecules are Y-shaped structures consisting of two identical light chains and two identical heavy chains that are paired by disulphide bonds to create three structural domains, two Fragment antigen-binding (Fab) and one Fragment crystallisable (Fc) fragments. The two Fab fragments have the variable (V) region at the amino terminus, which contributes to the antigen-binding. In contrast, the Fc fragment bears the constant (C) region that determines the antibody's isotype [302].

The Fc fragment is formed by a heavy chain dimer of the constant heavy 2 (CH2) and constant heavy 3 (CH3) segments. In contrast, the Fab fragment is a mixed light-heavy chain dimer of variable light (VL)-constant light (CL) paired with variable heavy (VH)-constant heavy 1 (CH1) segments. The high variability in the Fab fragment is created by V(D)J (heavy chain) or VJ (light chain) recombination and thus creating extensive diversity. Furthermore, the Fab fragment recognises antigen using its six complementarity-determining regions (CDR) loops (L1, L2, L3, H1, H2, and H3) loops. Because the CDR H3 is formed at the VDJ junction where there are deletions or insertions of extra nucleotide sequences, the CDR H3 is the most variable in length, sequence and structure (Figure 5.1) [303].

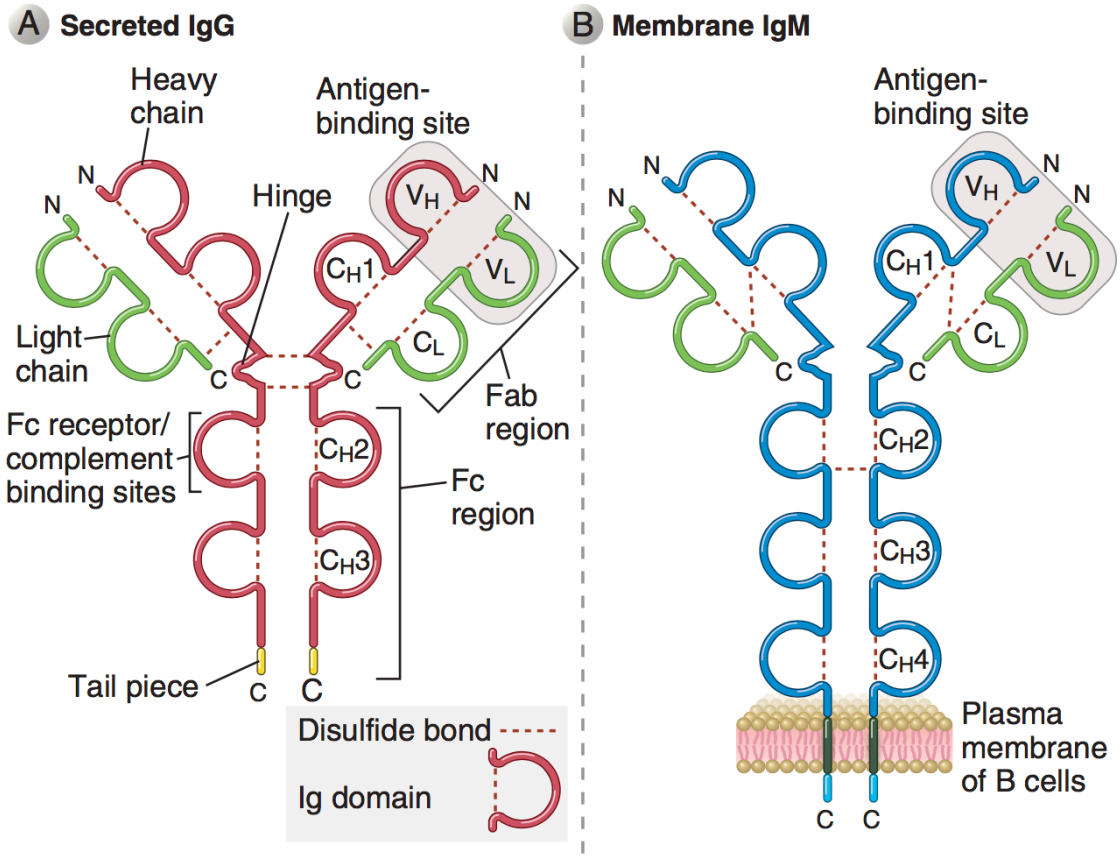


Figure 5:1: The representative structure of an immunoglobulin (Ig) -G antibody and a membrane-bound IgM molecule on the surface of a B cell. The Fab region is made up of the heavy (shown in blue and red) and light region (shown in green) and is the region that binds to antigens and is responsible for antibody neutralisation function against invading pathogens. The Fc portion is made up of the heavy chain and mediates non-neutralising functions such as antibody-dependent cellular phagocytosis [304].

The Fc portion is the part that mediates additional non-neutralising activities such as antibody-dependent cellular phagocytosis (ADCP), neutrophil phagocytosis (ADNP), complement deposition (ADCD) and cellular cytotoxicity (ADCC) [305]. Monocytes and neutrophils mediate antibody-dependent cellular phagocytosis while complement proteins in plasma are responsible for ADCD through the cell lysis of antibody-flagged infected cells. More specifically, complement component C3b activates the formation of membrane attack complexes leading to cell death or the recruitment of innate cells which will phagocytose the infected cells [304]. Natural killer (NK)

cells play a critical role in ADCC where infected cells are actively lysed via an antibody dependant mechanism [304]. These functions are guided by the antibody isotype class, which mainly relies on the cytokine milieu present in germinal centres (GCs) [306, 307]. The isotype class switching selects for constant regions, which, in turn, determines the scope of Fc effector functions [134]. Five antibody isotypes exist, IgA, IgD, IgE, IgG, and IgM. Isotypes IgD and IgGs have one variable and three constant domains while IgAs, IgEs and IgMs have one variable, four constant domains and an additional J-chain. The J chain allows the formation of dimers for IgEs and IgAs and pentamers for IgMs. Isotypes IgAs, IgDs, and IgGs are monomers [308].

Apart from isotype subclasses, the function of the Fc effector is modulated by their affinity for activating and inhibiting Fc receptors on innate cells and complement proteins [309]. For instance, activating Fc receptors include Fcγ 2a and Fcγ 3a, while Fcγ 2b is known to be an inhibiting Fc receptor. These activating Fc receptors are therefore capable of mediating pathogen clearance through phagocytosis by neutrophils, macrophages and monocytes, ADCC or direct lysis by natural killer cell activity and complement deposition among others. Lastly, Fc-mediated antibody functions are also dependent on the Fc domain glycosylation at the conserved site in the CH2 region, with both galactosylated and agalactosylated glycoforms being associated with improved human immunodeficiency virus (HIV) antiviral activity [306, 307, 310].

In human immunodeficiency virus (HIV) infection, Fc-mediated functions have been suggested to play a role in protection against HIV infection [240] and the control of viremia [305]. More convincing is that HIV elite controllers mount robust ADCC activity, that is thought to play a part in the suppression of viremia without antiretroviral treatment (ART) [143, 146, 311]. Moreover, coordinated Fc responses that involve the recruitment of various Fc effector functions have been

reported in elite controllers [146] and in HIV infected and vaccinated individuals [312]. More recently, it was shown that ADCP and ADCD are readily detectable as early as 6 months post-HIV infection and that, their early detection is predictive of the development of neutralisation breadth later in chronic HIV [134]. These mounting data strongly suggest that Fc-mediated function may be essential in the design of an effective antibody-based HIV vaccine.

Unlike Fc-mediated antibody functions that rise early in HIV infection [134], broadly neutralising antibodies (bnAbs) take years to be generated in adults and can only be detected in approximately 20% of HIV infected individuals [313]. These bnAbs exhibit high levels of somatic hypermutation in the complementarity determining regions due to extensive affinity maturation [314, 315] and are capable of neutralising most circulating HIV strains. It is plausible that the delay or lack of bnAbs development may be due to the immunodominance of non-conserved regions of the Envelope (Env) spike and the high variability in antigenic regions of the Env which favours viral immune escape. Additionally, the HIV spike is naturally unstable and tends to only express Env in conformations that favour the induction of non-neutralising antibodies over neutralising antibodies [316, 317].

The dominant epitopes targeted by bnAbs include the CD4 binding site (CD4bs), the V1-V2 apex, the V3-high mannose patch, the gp41 membrane-proximal external region (MPER) and the gp120/gp41 interface [318, 319]. More recently, another epitope targeted by bnAbs was discovered. The fusion peptide, which is located at the N terminus of the Env-gp41 subunit mainly aids in the entry of the virus into the host cell and is targeted by the bnAb N123-VRC34.01. The bnAb N123-VRC34.01 prevents viral entry into the host cells by hindering conformational changes in gp120 and gp41 subunits of Env that are necessary for cell entry [320]. Unlike in adults,

bnAbs in infants may develop earlier and target up to four distinct epitopes, with breadth achieved by a combination of these responses and hence enhancing the poly-clonality [321]. Currently, various epitopes on the HIV Envelop spike that are targeted by different monoclonal antibodies isolated have been mapped (Figure 5.2).

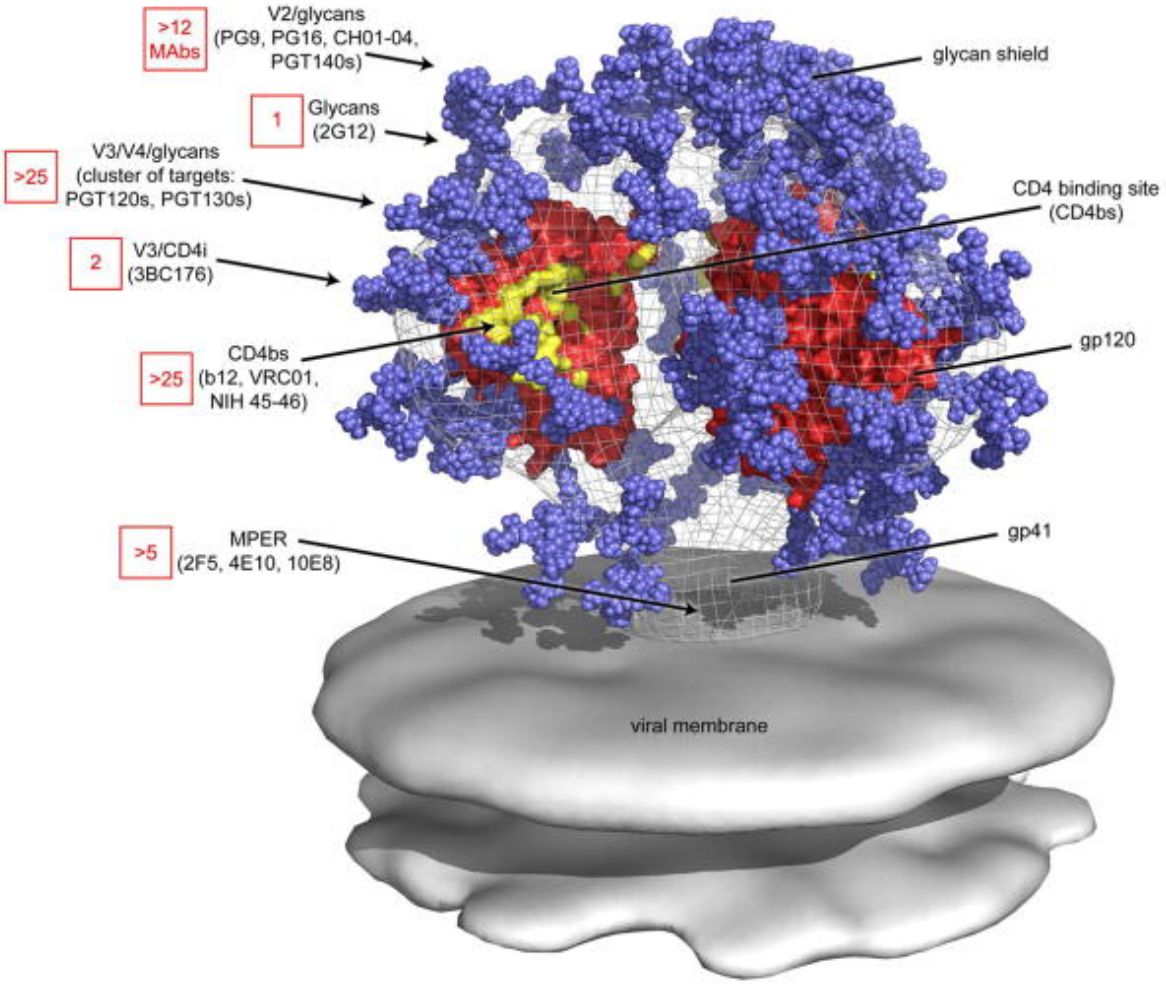


Figure 5:2: The structure and antibody recognition of the HIV Envelope spike. The approximate locations of epitopes targeted by monoclonal antibodies are shown by the arrows. Each red box represents the number of monoclonal antibodies targeting an epitope. Glycans are shown in purple. MPER; membrane-proximal external region. Adapted from [318].

In order to achieve a functional antibody-based HIV vaccine, assessing bnAbs may provide critical information that is crucial for optimal immunization strategies. Studies have suggested that bnAbs can protect against HIV acquisition in macaques [236, 322, 323] and in delaying viral rebound in HIV infected individuals off ART [132].

While the bnAbs only target few epitopes on the virus' Envelope, broadly neutralising monoclonal antibodies are resourceful in identifying key candidate epitopes more precisely [318]. It is thought that incorporating various targeted epitopes that generate bnAbs would be a good approach for vaccine design. However, all attempts have been futile. Attempts to design immunogens based on the CD4 binding site (CD4bs) have failed due to the specific directional targeting to the CD4bs required. The directional targeting is necessary to access epitopes that are occluded and can only be targeted through constrained angles of approach by the bnAbs. Similarly, targeting of the glycan epitope requires unique domain-exchanged configuration of this antibody that is necessary for the recognition of the glycan shield, which complicates the design [324].

The most promising target, MPER of Env gp41, has previously achieved some success in eliciting strong responses [325]. However, the proximity of the epitopes to the membrane has raised concerns in eliciting MPER antibodies [325, 326]. Additionally, MPER antibodies have been shown to be highly autoreactive and this may present problems moving forward in utilizing it as an immunogen in a vaccine [327].

Our knowledge of factors influencing the generation of bnAbs remains scarce. Several studies have suggested: high HIV viral load, low CD4+ T cell counts, subtype C infection and HLA-A*03(-) genotype [159], time after HIV infection [313], ethnicity [328], virologic characteristics

[329], increased frequencies of some subsets of T follicular helper (Tfh) cells and plasma levels of CXCL13 in early HIV infection [98, 99, 268] to play a role in bnAb development. Cellular frequencies of Tfh cells, critical in providing B cell help and key in the formation of GCs [210, 330], have been associated with the quality and quantity of anti-simian immunodeficiency virus (SIV) IgG antibodies in macaque models [331]. Similarly, the entire International Acquired immunodeficiency syndrome (AIDS) Vaccine Initiative (IAVI) protocol C cohort, Landais and others showed that the development of breadth was associated with high viral load, low total CD4+ T cell counts, virus subtype C infection and human leukocyte antigen (HLA) *A3(-) genotype [159].

Also, higher titres of total IgG and anti-Env binding antibodies have positively correlated with bnAbs generation. High viral load drives low CD4+ T cell counts, higher titres of anti-Env antibodies and may be determined by HLA type. It is plausible that the generation of bnAbs is influenced by the genotypic features of the transmitted virus. Indeed, some HIV subtype C features such as shorter V1–V4 loops have been shown to favour the development of bnAbs [332].

In this chapter, I determined the Fc and Fab functions of antibodies in HIV infected individuals. Specifically, I assessed ADCP, ADCD, ADNP and natural killer cells ability to express CD107α, macrophage inflammatory protein-1alpha (MIP-1α) and interferon-gamma (IFNγ) in plasma taken from HIV infected individuals over time (3-, 12-, 48- months post-HIV infection (PI)), using a gp120 coated bead assay at 3-, 12-, 48- months PI. For the Fab-mediated antibody function, plasma samples at 48 months PI were screened for broadly neutralising function against a 6 pseudovirus panel.

5.2: Objective

To determine Fc- and Fab-mediated antibody functions in HIV infection.

5.3: Results

5.3.1: Gating strategy for the Fc-mediated functions

Plasma samples were processed as described in chapter 2, section 9. All data were analysed on Flowjo version 10 (FlowJo LLC, Ashland, OR, USA). To gate on C3b deposition, gp120 coupled beads were gated on forward and side scatter plots, singlets selected, and positively labelled beads selected. The ability of antibodies to mediate complement deposition (ADCD) was then assessed by gating on the positively labelled beads that expressed C3b on the FITC fluorochrome (Figure 5.3). ADCD scores were determined by (% C3b deposition x MFI of deposited C3b).

In order to assess for neutrophil phagocytosis (ADNP), neutrophils were gated on the forward versus side scatter plot and were defined as CD3- CD14- CD66b+. Neutrophils that expressed FITC fluorochrome had phagocytised the gp120 FITC labelled beads. (Figure 5.4). The THP-1 cells were gated on the forward versus side scatter and ADCP determined by their ability to phagocytise FITC coupled beads (Figure 5.5). ADNP scores were determined by (% fluorescent bead phagocytosed x mean fluorescence intensity (MFI) of phagocytosed beads). ADCP scores were determined by ([% THP-1 cells that have taken up antigen coupled beads] x [MFI of THP-1 cells that have taken up antigen coupled beads])/10000

Antibody-dependent natural killer cell degranulation (ADNK) activity was determined through gating on natural killer cells on the forward versus side scatter plot (Figure 5.6). The natural killer cells were defined as CD3- CD16+ CD16- CD56+ CD56-. Their ability to degranulate was defined with the frequencies of the CD107α marker, ability to be activated by the interferon-gamma (IFNγ) marker and their ability to be chemo-attractive by the macrophage inflammatory protein-1alpha (MIP-1α) marker.

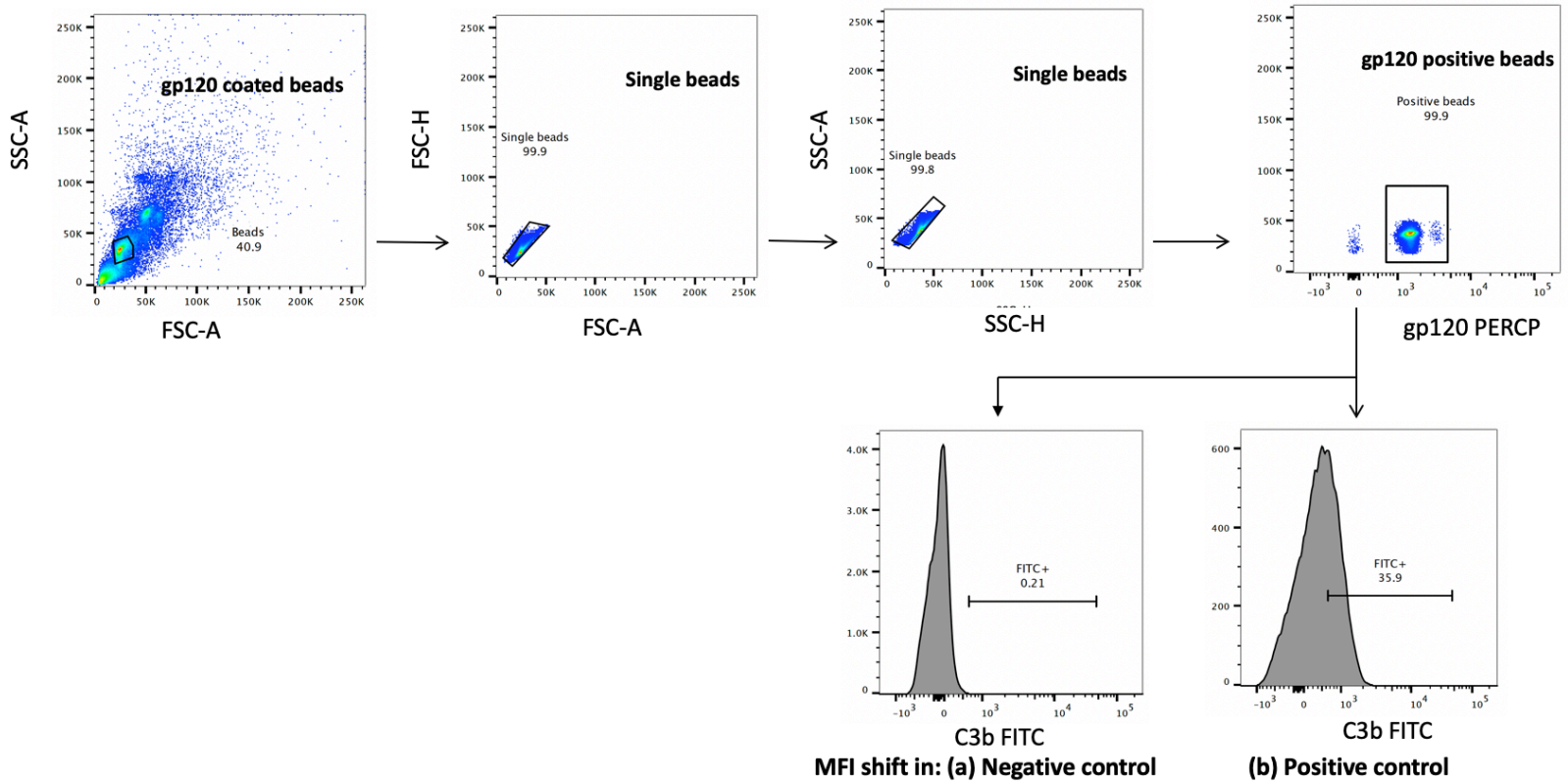


Figure 5:3: Representative gating strategy of ADCD. Beads were identified on forward scatter-side scatter plot. Within the beads gate, only gp120 positive beads were used to determine C3b deposition. The shift in the MFI and percentage of C3b deposited was used to give scores.

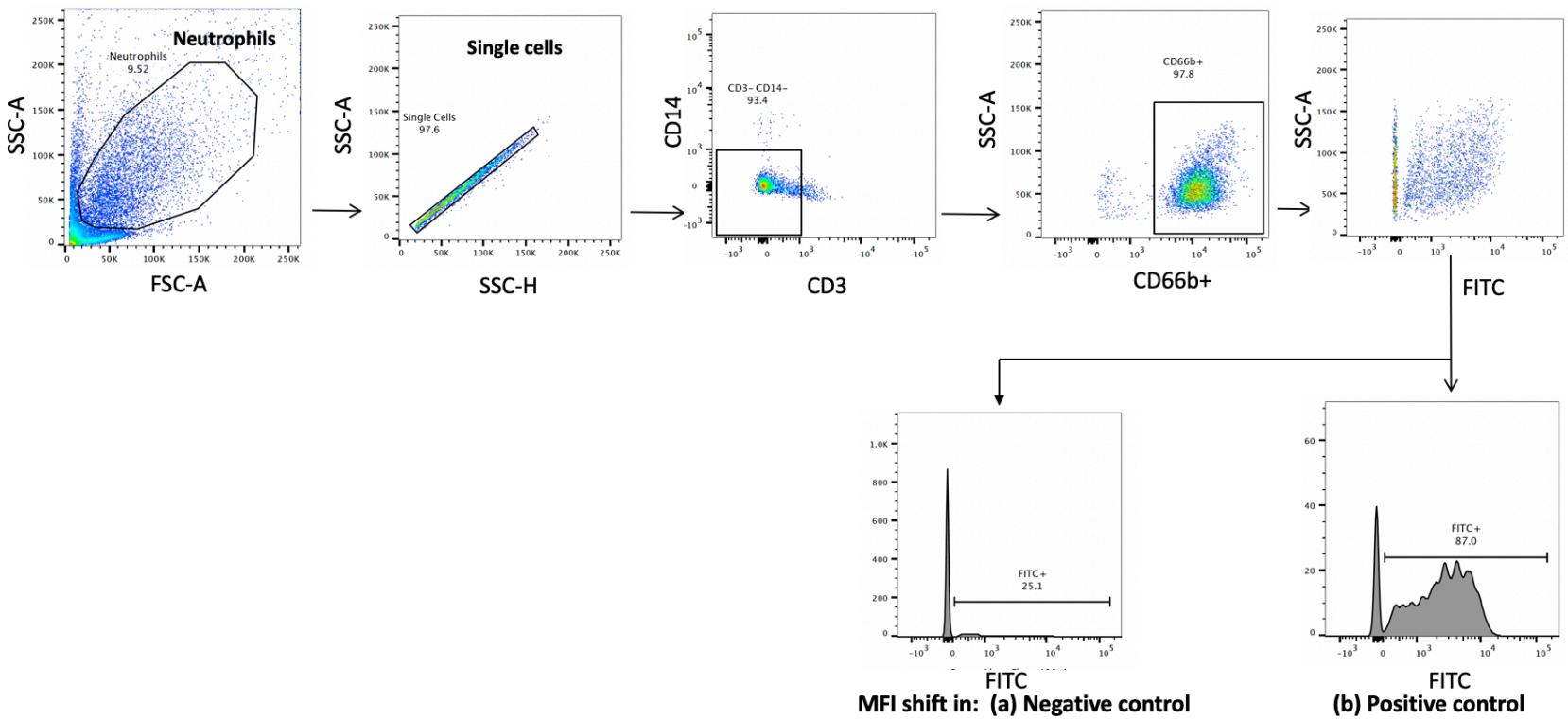


Figure 5:4: Representative gating strategy of ADNP. Neutrophils were identified on forward scatter-side scatter plot. Within the neutrophil gate, the markers CD3- CD14- CD66B+ was used to clean up the neutrophil population. Thereafter, gp120 FITC was used to determine which neutrophils took up a gp120 bead. Mean intensity fluorescence and the percentage of fluorescent bead phagocytosed was used to give scores.

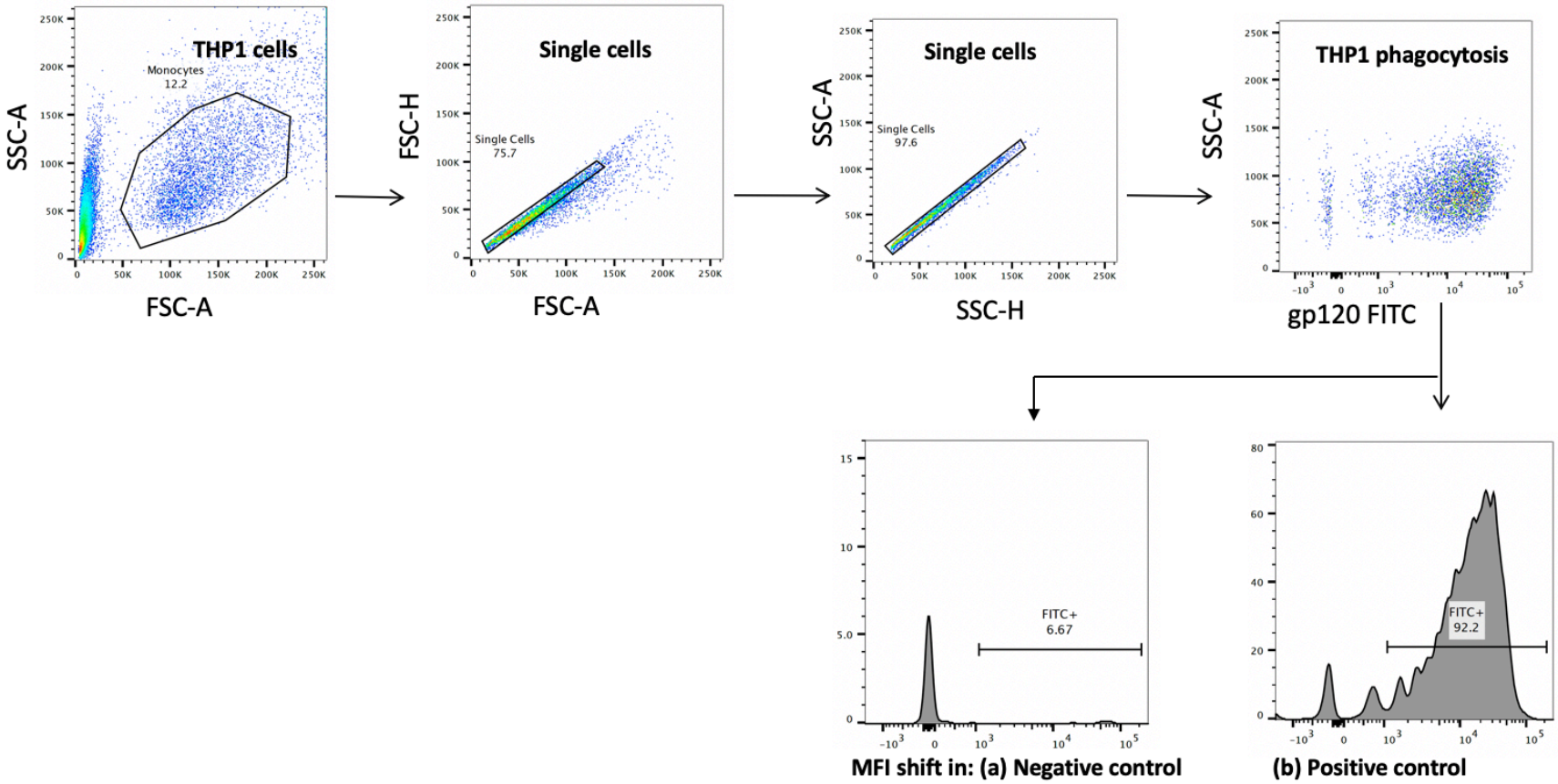


Figure 5:5: Representative gating strategy of ADCP using a THP1 cell line. Monocytes (THP1 cell line) were identified on forward scatter-side scatter plot. Within the monocytes gate, ADCP was determined by the ability of monocytes to phagocytose gp120 coated beads. Mean intensity fluorescence and the percentage of fluorescent bead phagocytosed was used to give scores.

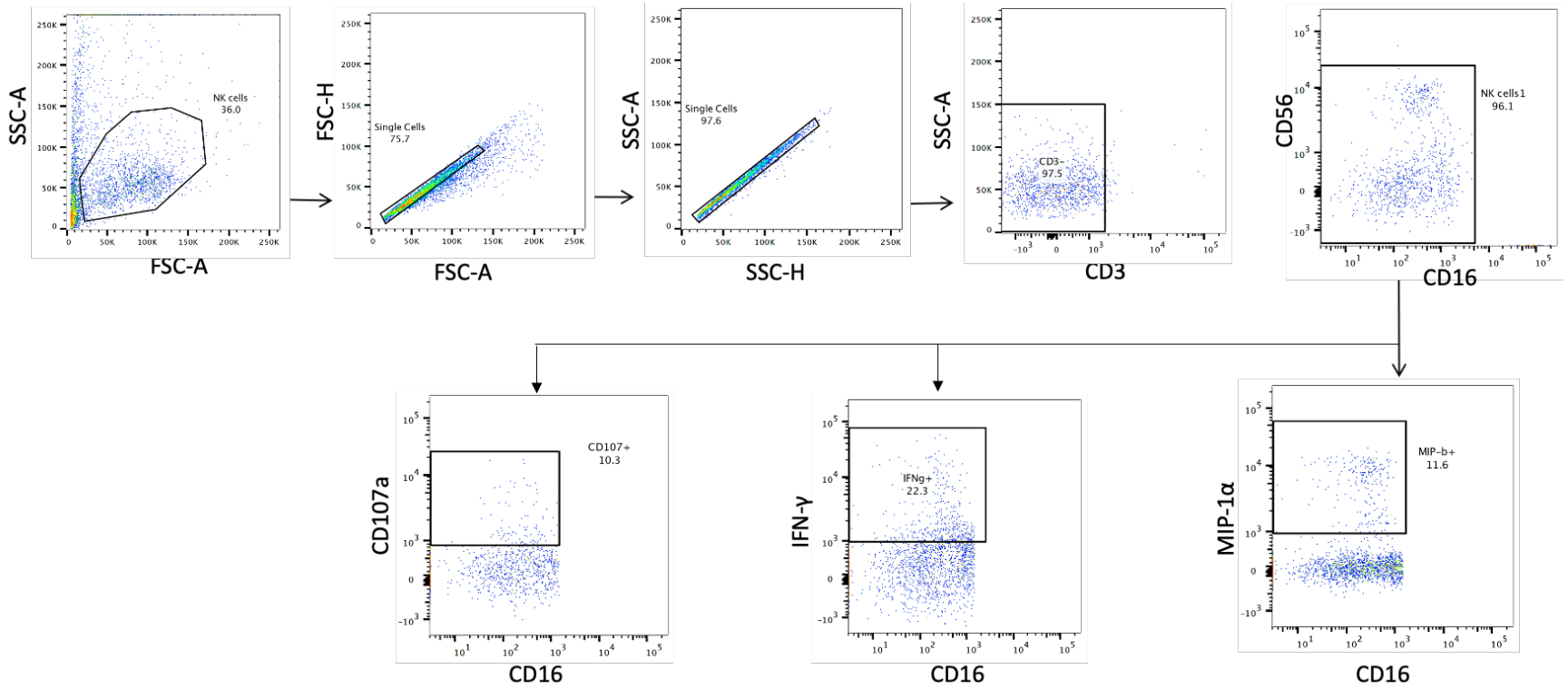


Figure 5:6: Representative gating strategy of ADNK assay. Purified natural killer cells were identified on forward scatter-side scatter plot. Within the natural killer cell gate, the markers CD3- CD56+ CD56- CD16+ CD16- were used to identify natural killer cells. Using this population, their ability to degranulate was defined with the CD107α marker, ability to be activated by the IFNγ marker and their ability to be chemoattractive by the MIP-1α marker. Cellular frequencies of these 3 markers were used to give ADNK scores.

5.3.2: Assessing the reproducibility of the Fc-mediated assays

All of the 53 HIV infected individuals included in this project were assessed for Fc-mediated functions. Plasma samples collected at approximately 3, 12- and 48- months post-HIV infection were used. Replicate assays were performed to confirm that results from these functional assays are reproducibility and reliable. The ADCD and ADCP assays were run on two different days while ADNK and ADNP assays were run on the same day but using blood from different donors. Reproducibility was assessed using rho values from the non-parametric Spearman's rank correlation. A correlation coefficient rho value of 0.3 - 0.5 was described as weak, 0.5 - 0.7 as moderate and > 0.7 as strong. The significance of the association was described with a p-value of less than 0.05 (Table 5.1).

There was a moderate to high correlation across all the assays conducted, confirming that the results are reliable. However, $ADNK_{IFN\text{-}\gamma}$ showed weak associations at the three time points. The proportion of $ADNK_{IFN\text{-}\gamma}$ detected per sample was low and may, therefore, interfere with the reproducibility. Neutrophil phagocytosis assay had the best reproducibility across all time points assessed.

Table 5.1: Correlation coefficients values of assays done on different days (ADCD and ADCP) or using different blood donors (ADNK and ADNP).

	Months PI	ADCD	ADCP	ADNP	CD107α	IFN-γ	MIP-1α
ADCD	3 months	0.4 (0.027)					
	12 months	0.59 (0.0002)					
	48 months	0.62 (0.007)					
ADCP	3 months		0.49 (0.007)				
	12 months		0.46 (0.0012)				
	48 months		0.78 (<0.0001)				
ADNP	3 months			0.7 (<0.0001)			
	12 months			0.81 (<0.0001)			
	48 months			0.9 (<0.0001)			
ADNK $_{CD107α}$	3 months				0.45 (0.01)		
	12 months				0.44 (0.01)		
	48 months				0.77 (<0.0001)		
ADNK $_{IFN-γ}$	3 months					0.31 (ns)	
	12 months					0.2 (ns)	
	48 months					0.52 (<0.0001)	
ADNK $_{MIP-1α}$	3 months						0.58 (0.003)
	12 months						0.77 (<0.0001)
	48 months						0.87 (<0.0001)

Plasma used was sampled at 3, 12- and 48- months post-HIV infection (PI). Spearman's rank correlation was used, and all p-values are shown in brackets in the cells. p-values <0.05 were considered significant. Antibody-dependent cellular phagocytosis; (ADCP), complement deposition (ADCD), neutrophil phagocytosis (ADNP) and natural killer cell degranulation ($ADNK_{CD107α}$), activation ($ADNK_{IFNγ}$) and ability to chemoattract ($ADNK_{MIP-1α}$). Ns; not significant, months PI; months post-HIV infection.

5.3.3: Fc-mediated antibody functions measured longitudinally at 3-, 12- and 48-months post-HIV infection

All the measured Fc-mediated functions, ADCD, ADCP, ADNP and ADNK were readily detectable at 3 months post-HIV infection in all individuals. These functions significantly increased over time except for ADCP, which peaked at 12 months PI but dropped by 48 months. The increase of ADCD activity may be due to the continuous antigenic stimulation that confers enhanced recruitment of complement and natural killer cells.

These results suggest that Fc-mediated responses arise early in infection, albeit low, but significantly increases by 12 months PI. Differences in Fc-mediated antibody functions were determined by one-way ANOVA (*Kruskal–Wallis test*) on the medians between the three time points at a 95% confidence interval. Differences in medians between paired time points were determined using the Mann-Whitney U test. The significance of each analysis was described with a p-value of less than 0.05 (Figure 5.7).

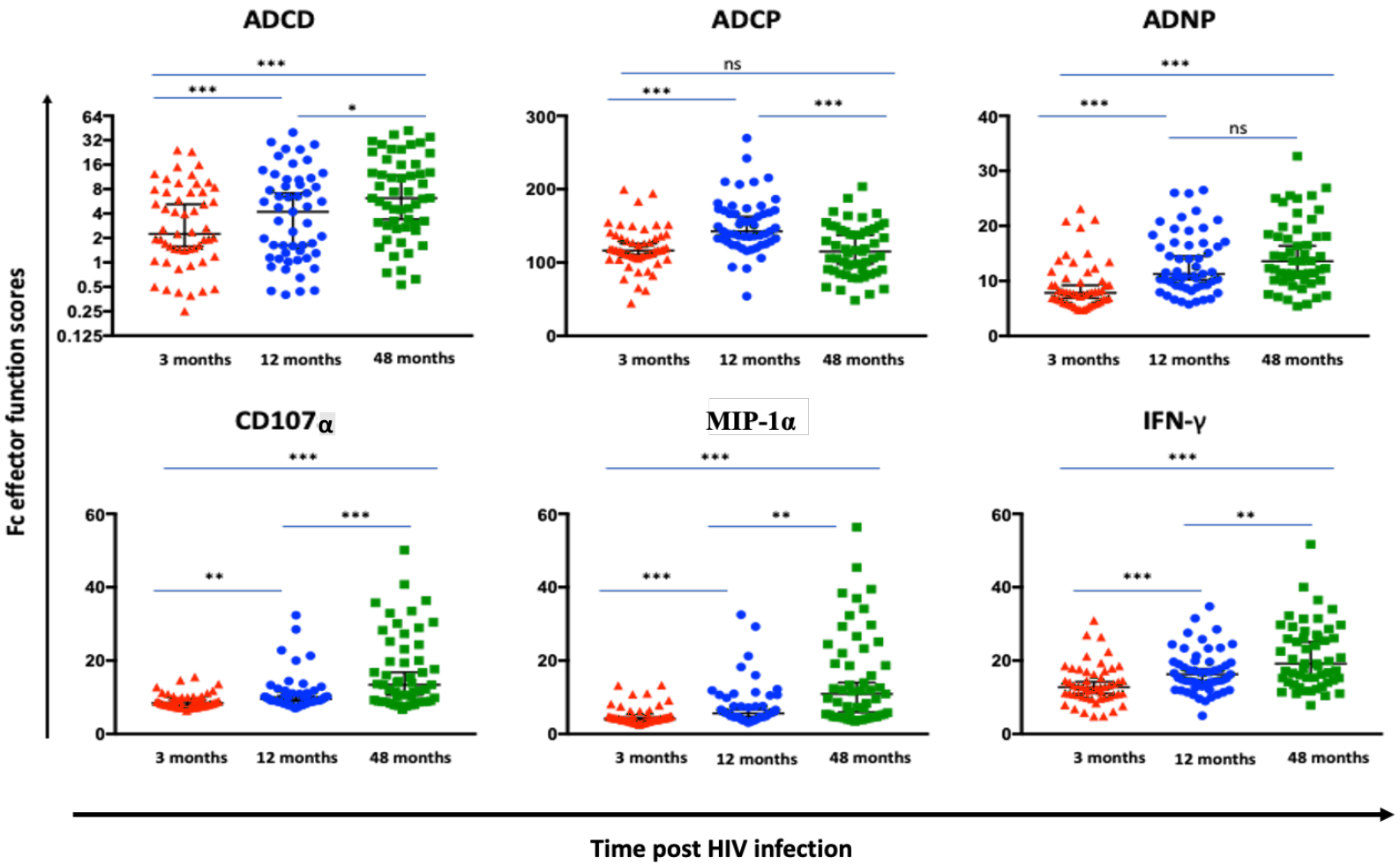


Figure 5:7: Fc effector function kinetics in HIV infection. Plasma samples collected at time point 1 (3 months), time point 2 (12 months) and time point 3 (48 months) PI were tested for antibody-dependent cellular phagocytosis (ADCP), complement deposition (ADCD), neutrophil phagocytosis (ADNP) and natural killer cell degranulation (CD107α), activation (IFNγ) and ability to chemoattract (MIP-1α). Differences between the time points were determined by the Mann-Whitney U test and significance established at p-value less than 0.05*, p-value < 0.01** and p-value < 0.001*.

5.3.4: Quality of the specific Fc-mediated functions generated in HIV

In order to test for the quality of individual Fc functions, each individual Fc function was standardized by calculating a Z score. The Z score was determined by subtracting the value of the individual Fc function score from the mean Fc function score and divided by the standard deviation of the mean. The Z score was determined by subtracting the value of the individual Fc function score from the mean Fc function score and divided by the standard deviation of the mean. This was calculated for each Fc-mediated antibody function. Quality Fc function was defined by a Z score greater than zero, while poor Fc function was defined by Z scores less than zero.

Study participants with Z-scores greater than zero, hence reflecting good quality, consistently maintained such responses over the three time points., Similarly those with Fc-mediated Z-scores less than zero maintained the poor responses as HIV disease progressed.

Of all Fc-mediated responses, more than 24 (45%) of participants had high ADNK function Z scores – measured by IFNγ secretion, across all time points. On the other hand, very few individuals had high secretion of MIP-1α, indicative of poor macrophage activation by HIV, with only 15 (28.3%) demonstrating high function at 12 months PI. The proportion of individuals with increasing individual Fc function across the 3 timepoints were generally maintained over time (Table 5.2).

Table 5.2: Proportion of individuals with quality specific Fc-mediated functions across the time points.

	Timepoint 1 (3 months PI)	**Timepoint 2 (12 months PI)**	**Timepoint 3 (48 months PI)**
ADCD	19 (35.8%)	18 (33.9%)	20 (37.7%)
ADCP	21 (39.62%)	23 (43.4%)	25 (47.2%)
ADNP	19 (35.8%)	22 (41.5%)	22 (41.5%)
ADNK$_{CD107\alpha}$	21 (39.62%)	13 (24.5%)	18 (33.9%)
ADNK$_{IFN\gamma}$	24 (45.3%)	27 (50.9%)	24 (45.3%)
ADNK$_{MIP\text{-}1\alpha}$	21 (39.62%)	15 (28.3%)	16 (30.2%)

The numbers and percentages of participants with high individual Fc-mediated functions; antibody-dependent cellular phagocytosis (ADCP), complement deposition (ADCD), neutrophil phagocytosis (ADNP) and natural killer cell (ADNK) - degranulation - (CD107α), activation - (IFNγ) and ability to chemoattract - (MIP-1α) stratified across sampling time points. The quality of individual Fc-mediated function was determined by calculating a Z score to standardize individual Fc-mediated scores on R studio as described above. The sample size and proportion of individuals with quality Fc effector functions are indicated by the numbers and percentages, respectively. PI; post-HIV infection.

A comparison of median HIV viral load measurements and CD4 T cells counts at the three time points between the individuals with good or poor-quality individual Fc-mediated functions was done. Quality ADCP function at 3 months PI was associated with significantly lower HIV viral copies/ml, 4567 [IQR, 46.5-24475] versus 97689 [IQR, 9153-462250], $p < 0.0001$ in individuals with poor ADCP responses (Mann-Whitney test). In addition, associations between HIV viral load measurements and CD4 T cells counts with Fc-mediated functions were done. ADCP function at 3 months PI negatively correlated with HIV viral load measurements at 3 months PI (Spearman's correlation rho=-0.38, p=0.0051) implying that ADCP may play a role in controlling early HIV

viremia (Figure 5.8). However, none of the Fc-mediated functions tested showed any association with CD4+ T cell counts and HIV viral load measurements.

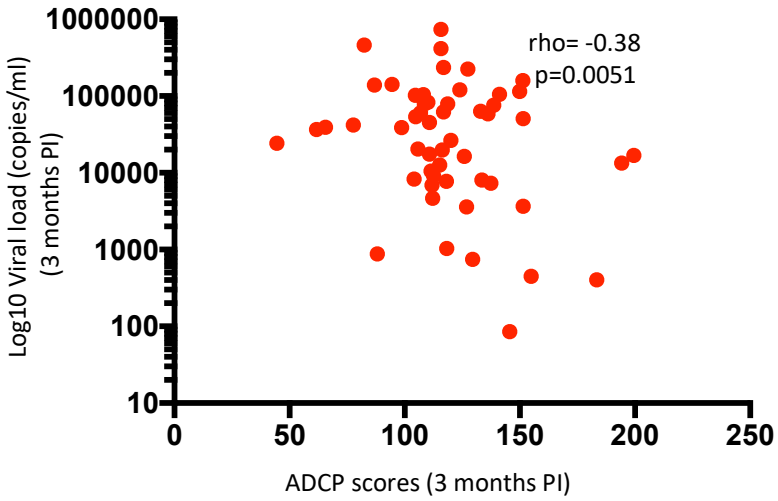


Figure 5:8: Correlation between ADCP and HIV viral load measurements at 3 months PI. Antibody-dependant complement deposition scores at 3 months post-HIV infection (PI) negatively correlated with HIV viral load measurements at 3 months PI. The y-axis shows HIV viral load copies/ml in log10 while the x-axis represents antibody-dependent cellular phagocytosis (ADCP) scores at 3 months PI. Significance was defined by $p < 0.05$ after conducting non-parametric Spearman's correlation. PI abbreviates "post-HIV infection".

5.3.5: Assessing for associations between different Fc-mediated functions in HIV

To check for the coordination between different Fc-mediated antibody functions, Spearman correlations were used. The various Fc-mediated antibody functions were significantly correlated in the course of HIV infection. The strongest correlation was observed between ADNP and $ADNK_{CD\ 107\alpha}$ (Spearman rho = 0.7, $p<0.001$), at 48 months PI. Generally, ADCP had the weakest association with the other Fc-mediated functions (Figure 5.8). At 3- and 12-months PI, $ADNK_{IFN\gamma}$ function poorly associated with all other Fc-mediated functions but moderately correlated with ADCD and ADNP functions at 48 months PI. Individuals who made good $ADNK_{IFN\gamma}$ function early in the infection maintained this throughout to 48 months PI.

Additionally, ADCD was significantly associated with the other ADNK functions ($ADNK_{CD\ 107\alpha}$ and $ADNK_{MIP\text{-}1\alpha}$ expression) across all time points except $ADNK_{IFN\gamma}$ at 3- and 12-months PI. Association of the different Fc-mediated functions suggested a coordinated functional response against HIV (Figure 5.9).

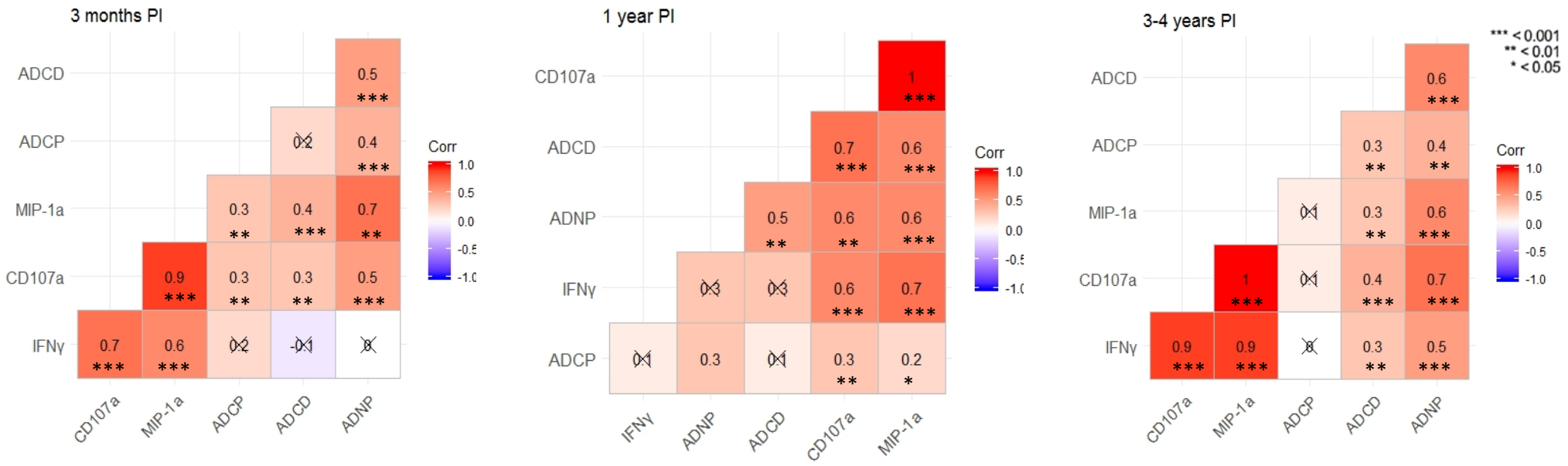


Figure 5:9: Correlation between the various Fc-mediated functions tested.

The matrix represents a pairwise comparison of Fc functions - antibody-dependent cellular phagocytosis (ADCP), complement deposition (ADCD), neutrophil phagocytosis (ADNP) and natural killer cell degranulation (CD107α), activation (IFNγ) and ability to chemoattract (MIP-1α), tested at a) 3 months, b) 1 year (12 months PI) and c) 3-4 years post-HIV infection (48 months PI). Colour coding represents the level of association with the strongest positive association shown in dark red while inverse correlations appear blue. P-values < 0.001 representing strong association (***), p<0.01 representing (**) and p<0.05 representing weak association (*). Test; Spearman's correlation.

5.3.6: Fc-mediated polyfunctionality in a longitudinal followed up HIV cohort

To establish the cumulative effect of the various Fc-mediated antibody responses per individual, an Fc polyfunctionality Z-scores was calculated for each participant at each sampling timepoint. The Z scores were calculated for each function, as described in Chapter 5, section 3.4 and were summed for each individual at each time point. Cumulative Z-scores with a value greater than zero indicated a well-coordinated function termed as "high Fc polyfunctional activity". In contrast, those with cumulative Z-scores less than zero indicated a poorly coordinated function termed as "poor Fc polyfunctionality".

At 3 months PI, of the 53 individuals, 35.8% (19/53) had Z-scores greater than 0, indicating that they mounted coordinated Fc-mediated antibody responses (Figure 5.10). It is plausible that this, together with the early CD8+ T cell responses may have played a part in bringing down the HIV viremia to a viral set point.

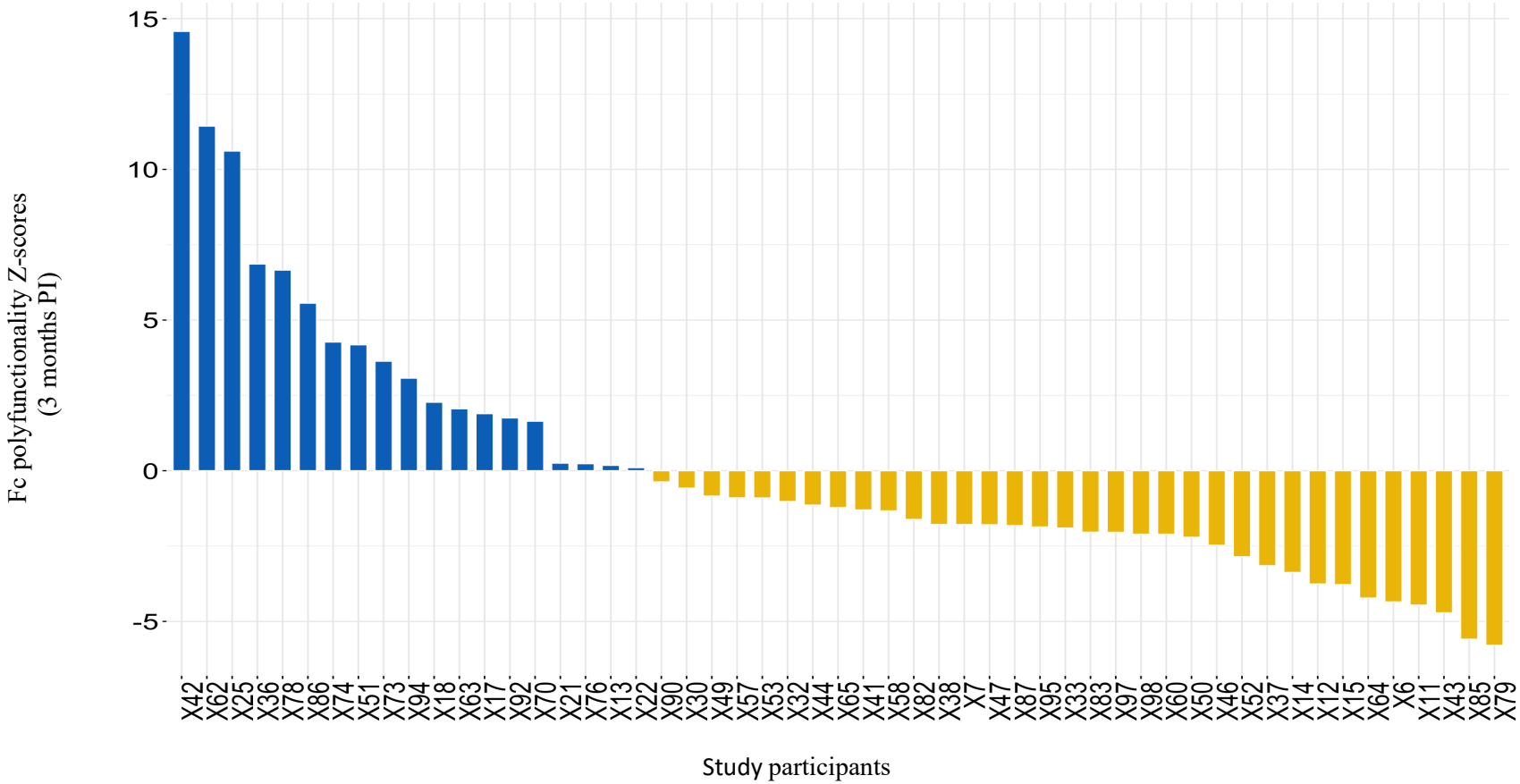


Figure 5:10: Fc polyfunctionality Z scores at 3 months post-HIV infection.

Fc polyfunctionality Z-score was determined by summing up individual Fc functions Z-scores (ADCP, ADCD, ADNP and ADNK (CD107α), activation (IFNγ) and ability to chemoattract (MIP-1α)) at 3 months PI. The x-axis represents the 53 individuals that are stratified by their polyfunctionality activity. The y-axis indicates polyfunctionality Z scores. Bars above zero (blue) indicate quality Fc polyfunctionality, while those below (gold) indicate poor Fc polyfunctionality. PI abbreviates “post-HIV infection”.

However, there was a decrease in the proportion of individuals who showed high Fc polyfunctionality at 12 months PI from 35.8% (19/53) to 30.2% (16/53) (Figure 5.11). This may be as a result of the immense viral escape, and the increasing numbers of HIV quasispecies that may deter quality Fc-mediated responses. Additionally, it may be due to the functional disruption of the B and Tfh cell compartments caused by direct and indirect effects of HIV. Such a functional disruption to the B and T cell compartments may lead to poor GCs activity and thus poor antibody function.

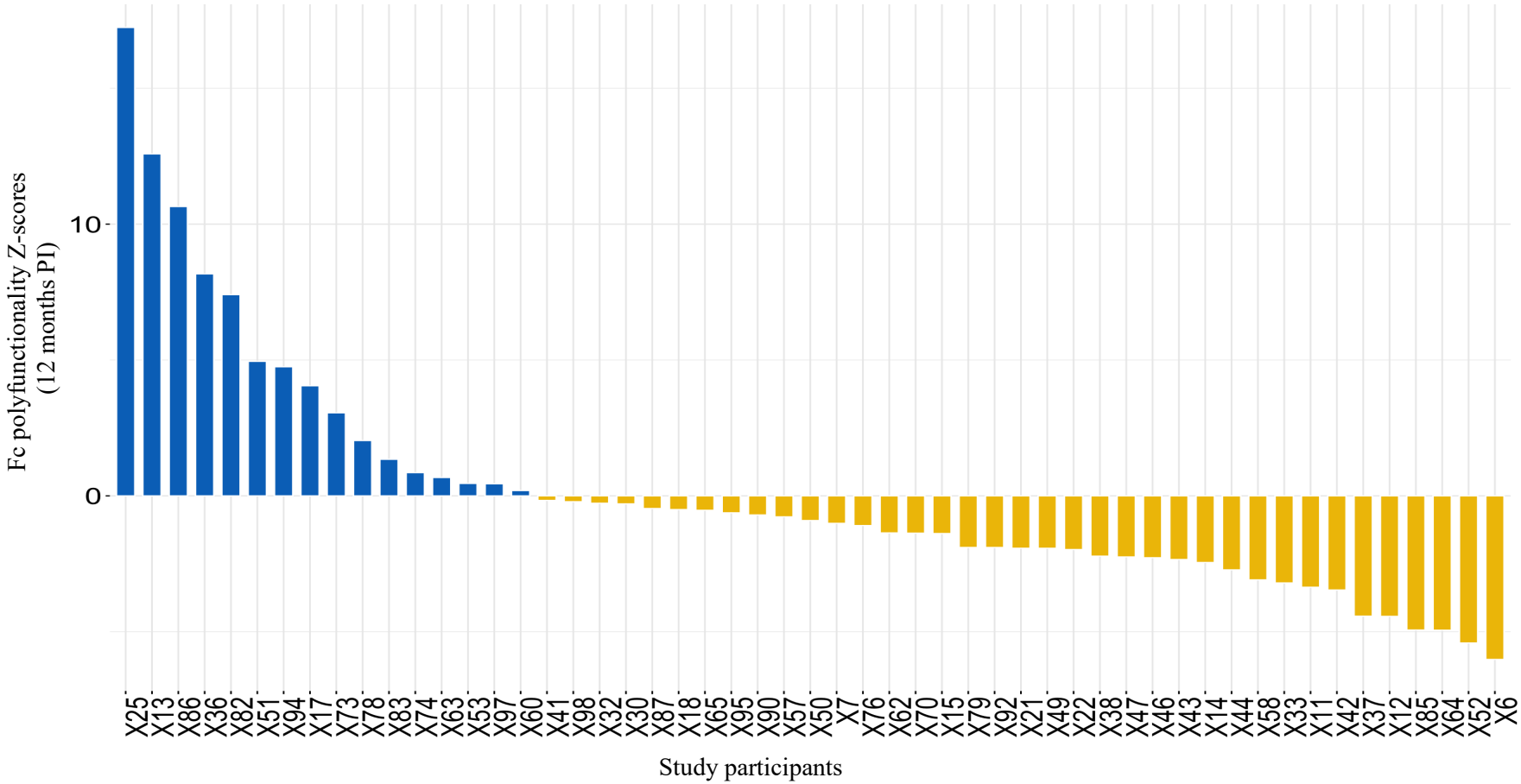


Figure 5:11: Fc polyfunctionality Z-scores at 12 months PI.

Fc polyfunctionality Z-score was determined by summing up individual Fc functions Z-scores (ADCP, ADCD, ADNP and ADNK (CD107α), activation (IFNγ) and ability to chemoattract (MIP-1α)) at 12 months PI. The x-axis represents the 53 individuals that are stratified by their polyfunctionality activity. The y-axis indicates polyfunctionality Z scores. Bars above the zero mark (blue) indicate quality Fc polyfunctionality, while those below (gold) indicate poor Fc polyfunctionality. PI abbreviates “post-HIV infection”.

However, at 48 months PI, the proportion of individuals with Fc polyfunctionality increased to 41.5% (22/53) (Figure 5.12). It is plausible that this increase in polyfunctionality activity was due to antibody maturity in response to higher HIV viral encounter at 48 months PI. Indeed, all individual Fc-mediated functions except ADNP had a significant increase in activity at 48 months PI.

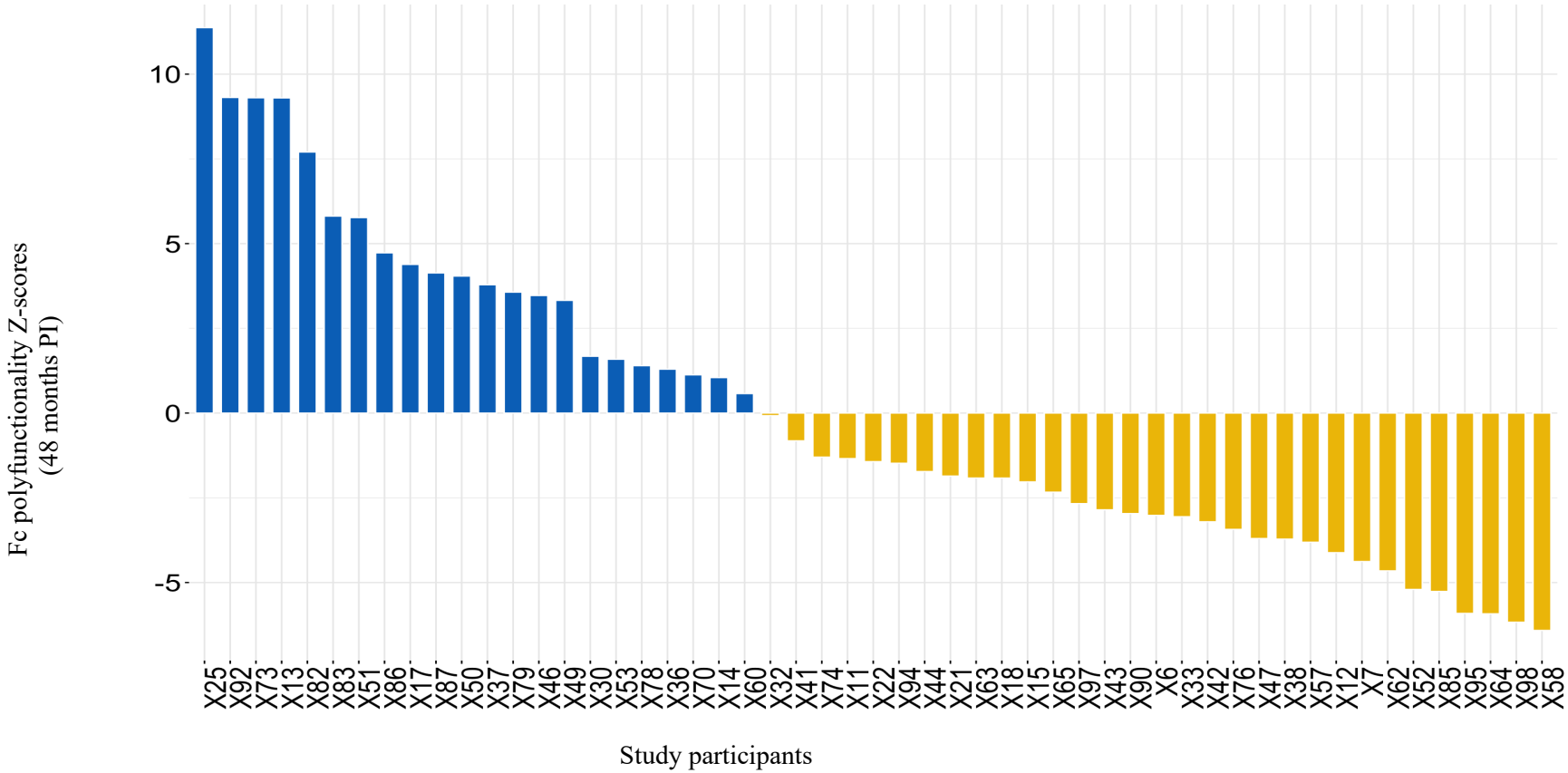


Figure 5:12: Fc polyfunctionality Z-scores at 48 months PI.

Individual Fc-mediated functions were standardized by calculating a Z-score as described above and the Fc polyfunctionality determined by the addition of the Z-scores for all 6 Fc-mediated functions (ADCP, ADCD, ADNP and ADNK (CD107α), activation (IFNγ) and ability to chemoattract (MIP-1α)) at 48 months PI. The x-axis represents the 53 individuals that are stratified by their polyfunctionality activity. The y-axis indicates polyfunctionality Z scores. Bars above the zero mark (blue) indicate quality Fc polyfunctionality, while those below (gold) indicate poor Fc polyfunctionality. PI abbreviates “post-HIV infection”.

5.3.7: Comparison of the levels of the markers of HIV disease progression in individuals with high versus low Fc polyfunctionality

The median values of the markers of HIV disease progression, CD4+ T cell counts and HIV viral load measurements taken at 3-, 12- and 48-months PI were compared in individuals with poor versus quality Fc polyfunctionality determined at similar time points (3-, 12- and 48-months PI).

No differences were observed in HIV viral load measurements between individuals with low versus high Fc polyfunctionality scores across the three time points. Similarly, individuals with quality Fc polyfunctionality did not preserve CD4+ T cell counts any better than those with poor Fc polyfunctionality at time points 3- and 12-months PI. However, individuals with poor Fc polyfunctionality had significantly higher median CD4+ T cell counts than those with quality Fc polyfunctionality at 48 months PI, 551 versus 349, $p=0.02$ (Mann-Whitney test) (Table 5.3). This finding may imply that higher viremia may enhance Fc function albeit the depletion of CD4+ T cell counts, with HIV disease progression [22].

Table 5.3: The effect of Fc polyfunctionality on the markers of HIV disease progression.

	Months PI	High Fc polyfunctionality-3 months P1	Low Fc polyfunctionality-3 months P1	p-value	High Fc polyfunctionality-12 months P1	Low Fc polyfunctionality-12 months P1	p-value	High Fc polyfunctionality-48 months PI	Low Fc polyfunctionality-48 months PI	p-value
Median HIV viral load (copies/ml)	3 months	38800 (1030-102000)	37950 (12075-87950)	0.31						
	12 months				18578 (3470-178500)	36250 (9648-95510)	0.51			
	48 months							35751 (8431-127,965)	22373 (6910-51076)	0.11
Median CD4+ T cell counts (cells/mm^3)	3 months	533 (283-689)	495 (383-628)	0.91						
	12 months				521 (321-605)	556 (435-765)	0.38			
	48 months							**349 (252-515)**	**551 (474-625)**	**0.02**

Cumulative Fc polyfunctionality Z-scores calculated in section 5.3.6 were used assessed for their effect on HIV disease progression as described by CD4+ T cell counts and HIV viral load measurements at 3-, 12- and 48- months PI. Using matching time points for Fc polyfunctionality scores and the markers of HIV disease progression, the differences in medians of CD4+ T cell counts and HIV viral load measurements between individuals with high- and low- Fc polyfunctionality was evaluated. In each cell, are medians and p-values. Significant differences are shown in bold and blue. Test; Mann-Whitney, PI; post infection. p-values <0.05 were considered significant.

5.3.8: Correlation between Fc polyfunctionality with the markers of HIV disease progression

While there was generally no significant differences in HIV viral load measurements and CD4+ T cell counts (except at 48 months PI) between individuals with quality and poor Fc polyfunctionality, high Fc polyfunctionality earlier on in the infection (3 months PI) was associated with reduced HIV viremia later on in the infection (12 months PI), Spearman rho= -0.529, p=0.0197. This may imply that high Fc polyfunctionality may play a role in controlling early HIV viremia (Figure 5.13).

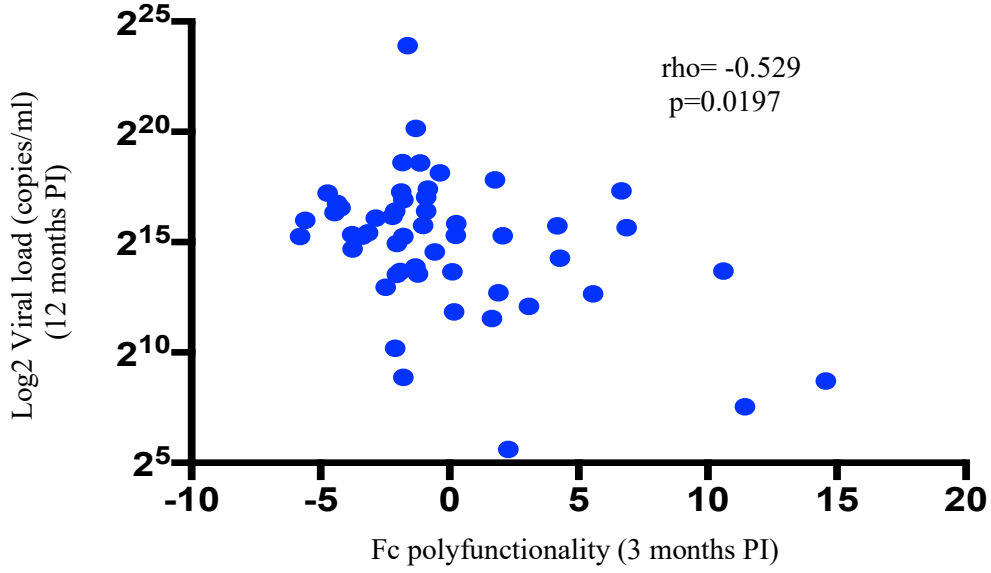


Figure 5:13: Correlation between HIV viral load measurements and Fc polyfunctionality. Fc polyfunctionality at 3 months PI inversely correlated with viral load measurements at 12 months PI. Indicated is the Spearman rho and the p-value. $p < 0.05$ was considered significant. The log10 of HIV viral load measurements and Fc polyfunctionality scores are shown on the y- and x-axes, respectively.

When assessing which particular individuals maintained high overall Fc polyfunctionality scores across all the 3 time points (sum of all individual Fc effector functions), 15.1% (8/53) of the participants had quality polyfunctionality. Additionally, 20.75% (11/53) individuals maintained high polyfunctionality at 3- and 12-months PI before these responses waned off by 48 months PI. Another 7.5% (4/53) individuals exhibited Fc polyfunctionality at 12 months PI and maintained these quality responses up to 48 months PI. In conclusion, high Fc polyfunctionality was rare in the cohort, and only 15.1% (8/53) of the study participants maintained these quality responses throughout the follow up period.

5.4: Antibody neutralisation function (Fab-mediated function)

5.4.1: Analysis of broadly neutralising function scores

Antibody neutralisation function was determined as described in chapter 2, section 10. From the raw data, the neutralising ability was defined using a previously reported approach [117] whereby, a neutralisation score is calculated based on the weighted average of log-transformed 50% neutralisation endpoint dilutions (IC50) across the pseudoviruses tested. In this case, a panel of 6 pseudoviruses, which were representative of a larger 105-virus panel [333] was used against plasma samples taken at 48 months PI and each individual got a neutralisation score accounting for breadth and potency.

Plasma breadth was accounted for by the number of pseudoviruses neutralised, while the potency was determined by the concentration of plasma required to neutralise the pseudoviruses. Presence of neutralising ability was defined as 50% inhibition of TZMbl cell line infection by an HIV

pseudovirus. A neutralisation score of ≥1 predicted ≥50% breadth on the larger panel. Scores of 0, 0 < 0.5, 0.5 <1 predicted 0%, 0.1-19.9%, 20-49.9% breadth respectively.

5.4.2: Validating the broadly neutralising antibody assay

Monoclonal antibodies VRC01 (targeting CD4 binding site), F105 (targeting CD4 binding site), PGT121 (targeting the V3 glycan), PGDM1400 (targeting the V2 glycan), PGT151 (targeting CD4 binding site and the fusion peptide) and 10E8 (targeting the membrane-proximal external region) were used as positive controls in this study. The negative control antibody used was the anti-dengue NS1 IgG[1], (DEN3.) These control antibodies were initially tested/validated for their ability to neutralise the 6 pseudoviruses (Table 5.4 and Figure 5.14) before the actual samples were tested.

Table 5.4: The neutralising ability of the control monoclonal antibodies against the 6-pseudovirus panel and controls in the assay set up the experiment.

	94UG103 (A)	**92TH021 (AE)**	**92BR020 (B)**	**JRCSF (B)**	**IAVIC22 (C)**	**93IN905 (C)**	**MN (B)**	**MLV**
Den3	0.611	0.141	3.664	21.410	0.012	0.195	0.031	1.048
F105	8.367	2.428	0.002			0.001	2.340	1.070
VRC01	0.011	0.010	0.003	0.011	0.008	0.003	0.001	2.569
PGT121	0.034	2.500	~ 9.5e-006	0.001	~ 4.0e-008	0.435	0.051	0.462
PGDM1400	0.01166	~4.1e-008	6.27	0.001	0.003	0.001	0.004	0.832
PGT151	0.066	0.088	2.2e-05	0.001	~ 2.3e-008	~ 145817	0.019	
10E8	0.9	0.057	3.584	0.115	0.028	0.007	0.001	~ 187

The table shows monoclonal antibodies tested against the pseudoviruses. The 50% inhibitory dose (ID50) concentrations of VRC01, F105, PGT121, PGDM1400, Den3, PG151 and 10E8 required to neutralise 94UG103 (A), 92TH021 (AE), 92BR020 (B), JRCSF (B), IAVIC22 (C) and 93IN905 (C) pseudoviruses are shown in the cells. The lower the ID50 value, the more potent the monoclonal antibody is, but this has to be confirmed with the dose-response curve illustrated in Figure 5.14. Blank cells represent analyses where the ID50 values could not be computed on Pad Prism. Test; nonlinear regression- inhibitor versus response (3 parameters). GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to analyse the data.

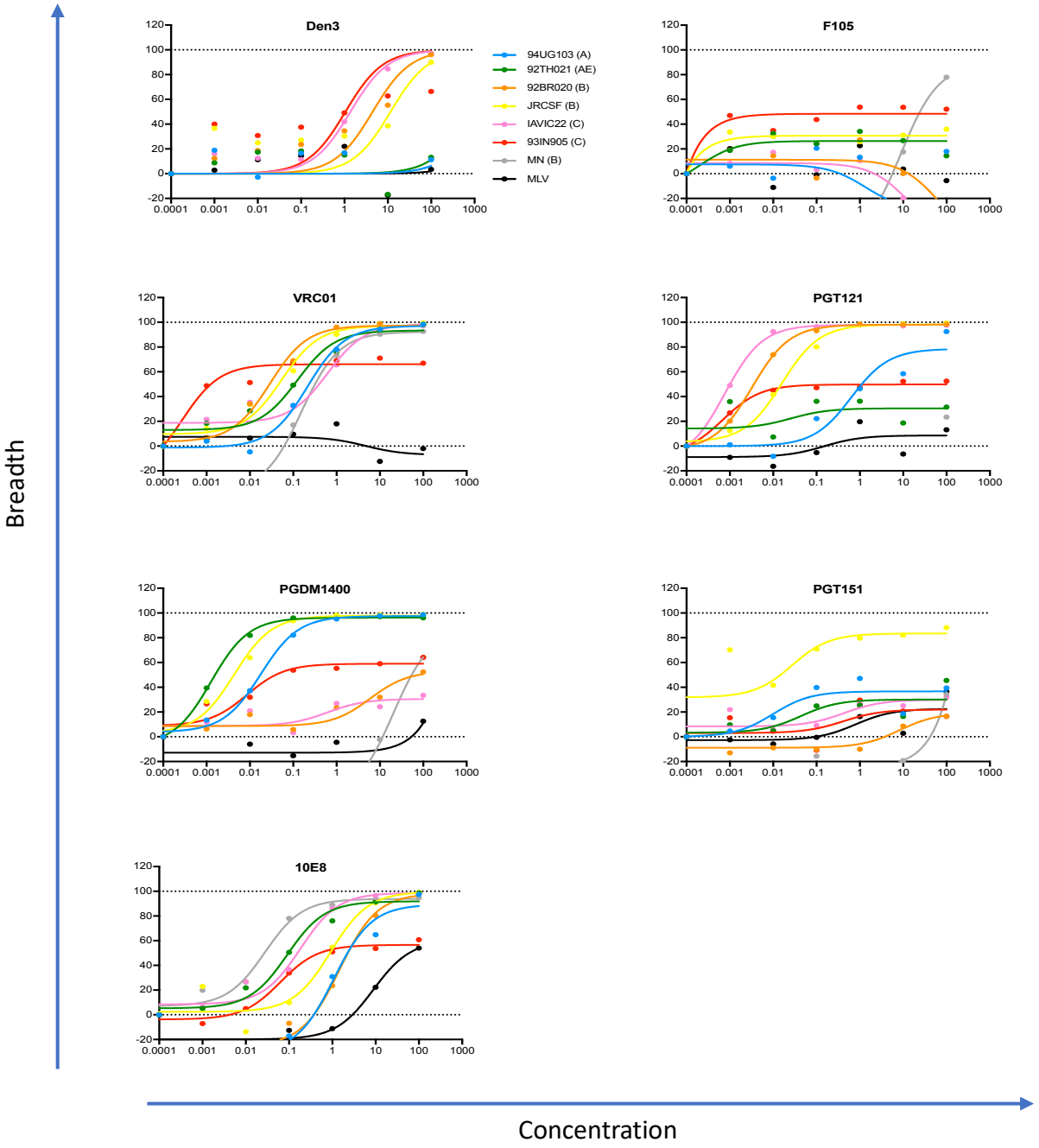


Figure 5:14: The neutralisation curves of the monoclonal antibodies used in the assay validation. The neutralisation curves for the of monoclonal antibodies VRC01, F105, PGT121, PGT151, PGDM1400, PG151 and 10E8 upon running the assay are shown. Each curve is colour coded to represent the pseudoviruses 94UG103 (A), 92TH021 (AE), 92BR020 (B), JRCSF (B), IAVIC22 (C), 93IN905 (C), MN (B) and MLV and is shown in the provided key. As expected, all curves but the black one (MLV pseudovirus) showed a dose-response effect, denoting successful neutralisation. Similarly, Den 3 failed to neutralise any of the pseudoviruses. The y-axis represents neutralisation breadth, while the x-axis represents control antibodies concentrations. Each monoclonal antibody is labelled at the top of its graph. Test; nonlinear regression- inhibitor versus response (3 parameters). GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to analyse the data.

5.4.3: Development of broadly neutralising antibody responses in chronic HIV

After running the initial assay set up to validate the neutralising ability of the monoclonal antibodies, the plasma samples were run together with the monoclonal antibodies to determine the neutralisation breadth of each plasma sample. As expected, the broad and potent monoclonal antibodies were able to neutralise all the 6 pseudoviruses in the panel but not the MLV pseudovirus (Table 5.5 and Figure 5.15). Furthermore, these monoclonal antibodies displayed high potency with very low concentrations required to achieve 50% breadth. Also, and as expected, the negative control antibody Den 3 failed to neutralise any of the pseudoviruses tested.

Table 5.5: Neutralising ability of the control monoclonal antibodies against the 6 pseudovirus panel and controls.

	94UG103 (A)	**92TH021 (AE)**	**92BR020 (B)**	**JRCSF (B)**	**IAVIC22 (C)**	**93IN905 (C)**	**MN (B)**	**MLV**
Den3			1.661	18.77	0.020	0.087		
F105			25.49	13446		0.114	9.688	
VRC01	0.012	0.0124	0.004	0.011	0.011	0.004	0.001	
PGT121	0.034	45.04	0.001	0.001	0.001	0.562	6.302	
PGDM1400	0.007	0.001	120.1	0.001	131	0.002	64.67	
PGT151	0.011	4.316	0.001	0.001	0.001	117.9	0.560	
10E8	0.691	0.098	1.615	0.195	0.017	0.007	0.001	143.8

The 50% inhibitory dose (ID50) concentrations of monoclonal antibodies VRC01, F105, PGT121, PGDM1400, Den3, PG151 and 10E8 required to neutralise 94UG103 (A), 92TH021 (AE), 92BR020 (B), JRCSF (B), IAVIC22 (C) and 93IN905 (C) during the assay. As expected, all the monoclonal antibodies neutralised the MN clade B virus, which is a tier 1 pseudovirus. In contrast, none of the control antibodies was able to neutralise the Murine retrovirus (MLV) pseudovirus. All values presented in the table are neutralisation titres [50% inhibitory dose (ID50)]. The lower the value, the more potent the monoclonal is, but this has to be confirmed with the dose-response curve illustrated in Figure 5.15. HIV clades are indicated in brackets on the top row. Blank cells represent analyses where the ID50 values could not be computed on GraphPad Prism7. Test; nonlinear regression- inhibitor versus response (3 parameters). GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to analyse the data.

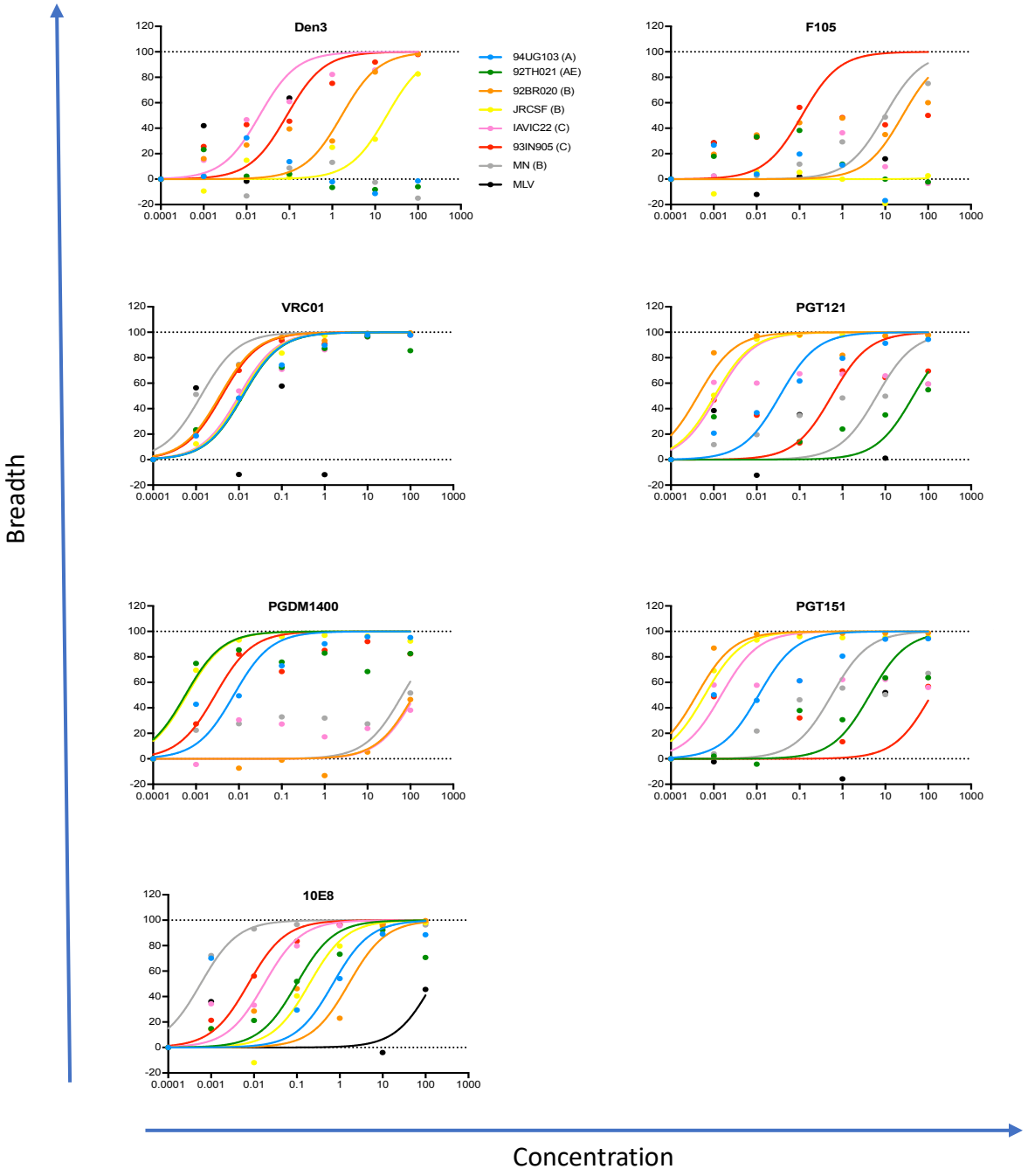
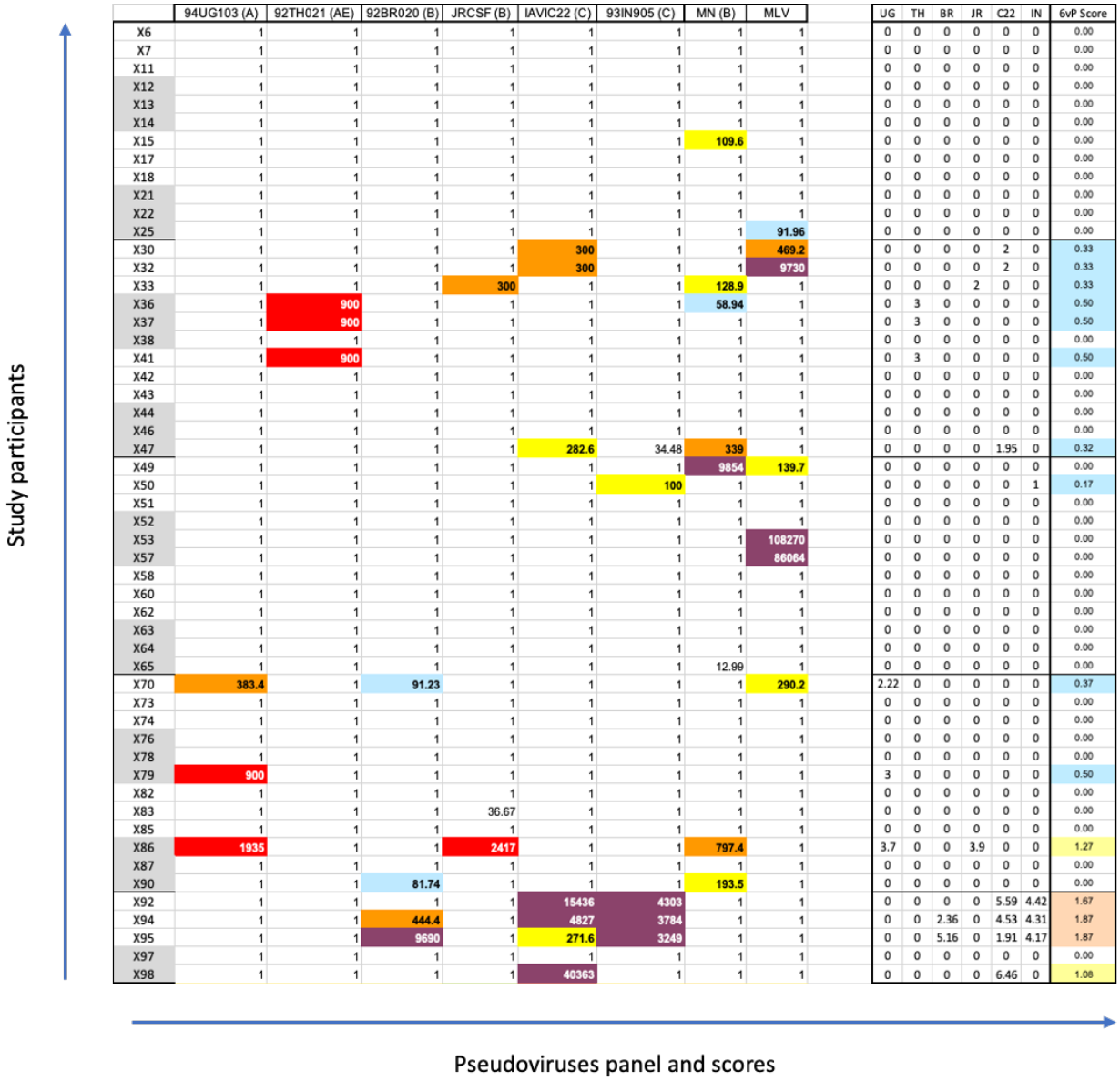


Figure 5:15: The neutralisation curves for the control monoclonal antibodies used in the experiments. The neutralisation curves for the of monoclonal antibodies VRC01, F105, PGT121, PGT151, PGDM1400, PG151 and 10E8 upon running the assay. Each curve is colour coded to represent the pseudoviruses 94UG103 (A), 92TH021 (AE), 92BR020 (B), JRCSF (B), IAVIC22 (C), 93IN905 (C), MN (B) and MLV and are shown in the provided key. As expected, all curves but the black one (MLV pseudovirus) showed a dose-response effect, denoting successful neutralisation. Likewise, Den 3 failed to neutralise any of the pseudoviruses. The y-axis represents neutralisation breadth while the x-axis represents control antibodies concentrations. Each monoclonal antibody is labelled at the top of its graph. Test; nonlinear regression-inhibitor versus response (3 parameters).

On assessing the 53 plasma samples, neutralising breadth was found to be rare in this cohort. This is consistent with the literature, where the development of breadth has been shown to be rare and to develop later on in HIV infection [313].

Only 28.3% (15/53) of the study individuals had breadth > 0% (neutralising any of the 6 pseudoviruses). Of the 15 individuals who could neutralise any pseudovirus, only 33.3% (5/15) of participants had a score >1 (>50% breadth/ neutralised more than 3 of the viruses). Another 26.7% (4/15) of the study individuals displayed moderate breadth (score ≥0.5 and <1) while 26.7% (4/15) of the study individuals showed low neutralisation breadth (0 < score < 0.5). A majority of study participants, 71.2%, (38/53) displayed no breadth on the 6-virus panel (Figure 5.16, 5.17 and Appendix 8).



	94UG103 (A)	92TH021 (AE)	92BR020 (B)	JRCSF (B)	IAVIC22 (C)	93IN905 (C)	MN (B)	MLV	UG	TH	BR	JR	C22	IN	6vP Score
X6	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X7	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X11	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X12	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X13	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X14	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X15	1	1	1	1	1	1	109.6	1	0	0	0	0	0	0	0.00
X17	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X18	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X21	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X22	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X25	1	1	1	1	1	1	1	91.96	0	0	0	0	0	0	0.00
X30	1	1	1	1	300	1	1	469.2	0	0	0	0	2	0	0.33
X32	1	1	1	1	300	1	1	9730	0	0	0	0	2	0	0.33
X33	1	1	1	300	1	1	128.9	1	0	0	0	2	0	0	0.33
X36	1	900	1	1	1	1	58.94	1	0	3	0	0	0	0	0.50
X37	1	900	1	1	1	1	1	1	0	3	0	0	0	0	0.50
X38	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X41	1	900	1	1	1	1	1	1	0	3	0	0	0	0	0.50
X42	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X43	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X44	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X46	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X47	1	1	1	1	282.6	34.48	339	1	0	0	0	0	1.95	0	0.32
X49	1	1	1	1	1	1	9854	139.7	0	0	0	0	0	0	0.00
X50	1	1	1	1	1	100	1	1	0	0	0	0	0	1	0.17
X51	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X52	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X53	1	1	1	1	1	1	1	108270	0	0	0	0	0	0	0.00
X57	1	1	1	1	1	1	1	86064	0	0	0	0	0	0	0.00
X58	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X60	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X62	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X63	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X64	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X65	1	1	1	1	1	1	12.99	1	0	0	0	0	0	0	0.00
X70	383.4	1	91.23	1	1	1	1	290.2	2.22	0	0	0	0	0	0.37
X73	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X74	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X76	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X78	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X79	900	1	1	1	1	1	1	1	3	0	0	0	0	0	0.50
X82	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X83	1	1	1	36.67	1	1	1	1	0	0	0	0	0	0	0.00
X85	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X86	1935	1	1	2417	1	1	797.4	1	3.7	0	0	3.9	0	0	1.27
X87	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X90	1	1	81.74	1	1	1	193.5	1	0	0	0	0	0	0	0.00
X92	1	1	1	1	15436	4303	1	1	0	0	0	0	5.59	4.42	1.67
X94	1	1	444.4	1	4827	3784	1	1	0	0	2.36	0	4.53	4.31	1.87
X95	1	1	9690	1	271.6	3249	1	1	0	0	5.16	0	1.91	4.17	1.87
X97	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0.00
X98	1	1	1	1	40363	1	1	1	0	0	0	0	6.46	0	1.08

Figure 5:16: The calculation to determine the evolution of breadth in a chronically HIV infected cohort.

The 50% inhibitory concentration (IC50) values for 94UG103 (A), 92TH021 (AE), 92BR020 (B), JRCSF (B), IAVIC22 (C), 93IN905 (C), MN (B) and MLV are shown on the left side of the figure. The higher the IC50 value, the more potent the plasma sample is, but this has to be confirmed with a dose-response curve illustrated in Figure 5.14. On the right are the calculated neutralisation scores against the 6 HIV pseudoviruses tested. Breadth was accounted for by the number of pseudoviruses neutralised, while potency was determined by the concentration of plasma required to neutralise the pseudovirus. Overall neutralising ability was defined by establishing a neutralisation score based on the weighted average of log-transformed 50% neutralisation endpoint dilutions (IC50) across the pseudoviruses tested. The higher the score, the higher the breadth and potency of neutralisation. The left-most column represents study participants while the right-most column represents neutralisation breadth scores based on the 6 pseudoviruses panel (6vP). The top row represents the pseudoviruses used in the assay. Test; nonlinear regression- inhibitor versus response (3 parameters). GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to analyse the data.

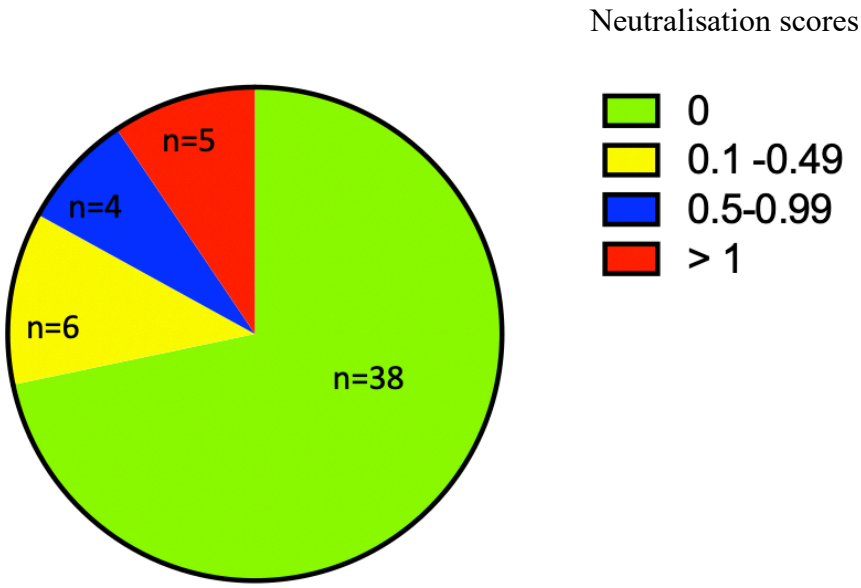


Figure 5:17: The proportions of individuals with and without neutralisation breath.

Plasma samples from HIV infected individuals collected at 48 months PI were tested for neutralising ability on a 6-virus panel. Neutralisation scores were calculated as outlined in section 5.4.1. The key represents neutralisation scores and its colour coded with the provided key. Test; the fraction of total on GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

A closer look at examples of neutralisation curves plotted as breadth versus concentration for the monoclonal antibody VRC01, and representative neutralisation curves plotted as breadth versus plasma dilution for a non-neutraliser ("X7") and a neutraliser ("X86") are shown in Figure 5.18.

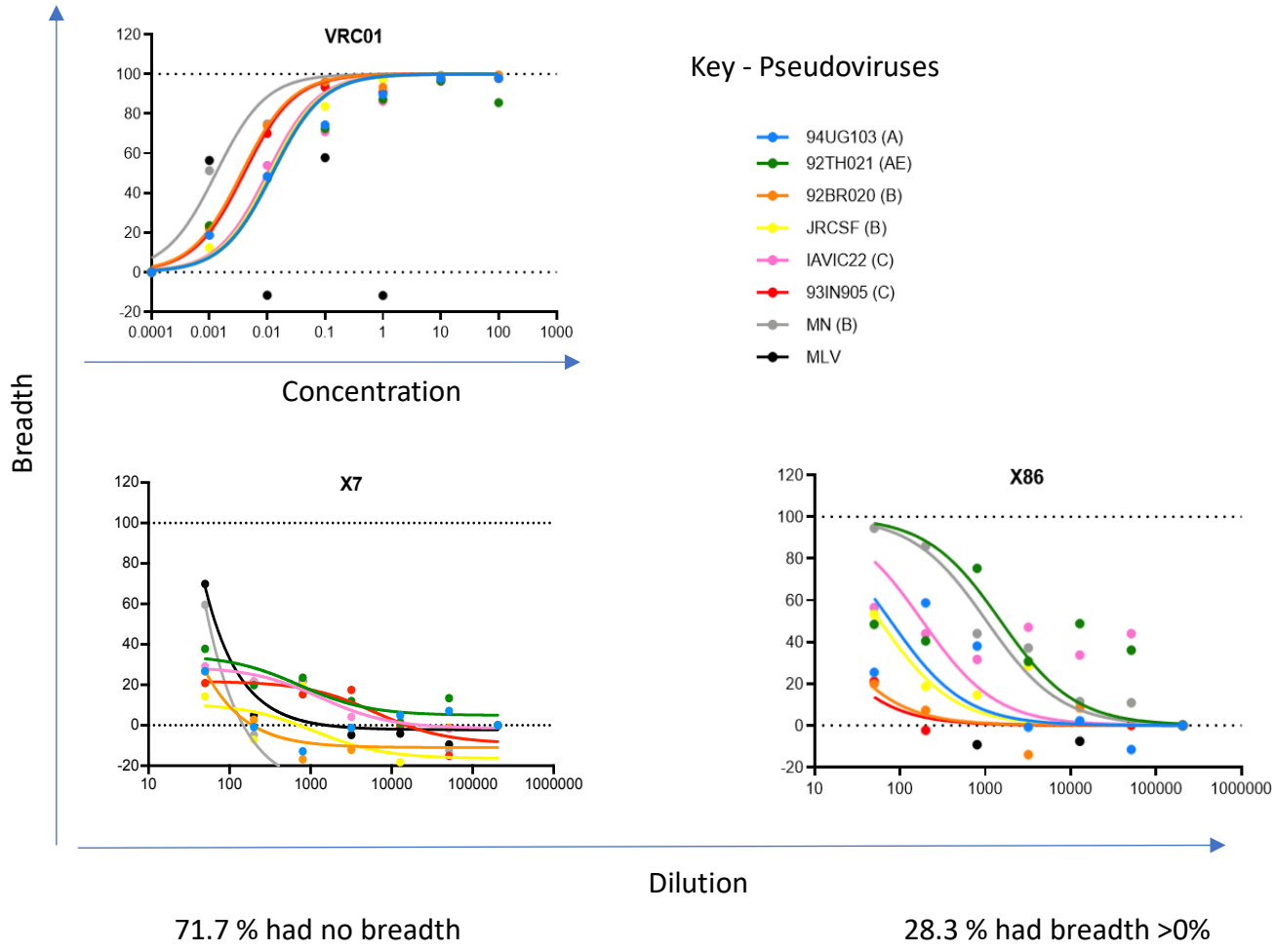


Figure 5:18: The neutralisation curves of plasma samples tested for breadth development.

The neutralisation curves for the broad and potent VRC01 antibody and plasma samples from a neutraliser (X86) and a non-neutraliser (X33). All the pseudoviruses are colour-coded and shown in the key. Each neutralisation curve is also colour-coded matching pseudoviruses. The y-axis represents breadth while the x-axis for the monoclonal VRC01 represents concentration, while the x-axis for the representative samples represent dilutions. Test; nonlinear regression- inhibitor versus response (3 parameters). GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to analyse the data.

5.4.4: The proportion of individuals neutralising pseudoviruses from a specific clade

Although this cohort was primarily infected with HIV clade A as described in chapter 2 section 1, most of the neutralisers showed some neutralisation activity against viruses from HIV clade C (IAVIIC22 and 93IN905). The pseudoviruses IAVIC22 was neutralised by 46.7% (7/15), while 93IN905 was neutralised by 26.7% (4/15) of the neutralisers. The other remaining 4 pseudoviruses were either neutralised by either 2, 3 or 4 of the neutralisers as highlighted (Figure 5.19).

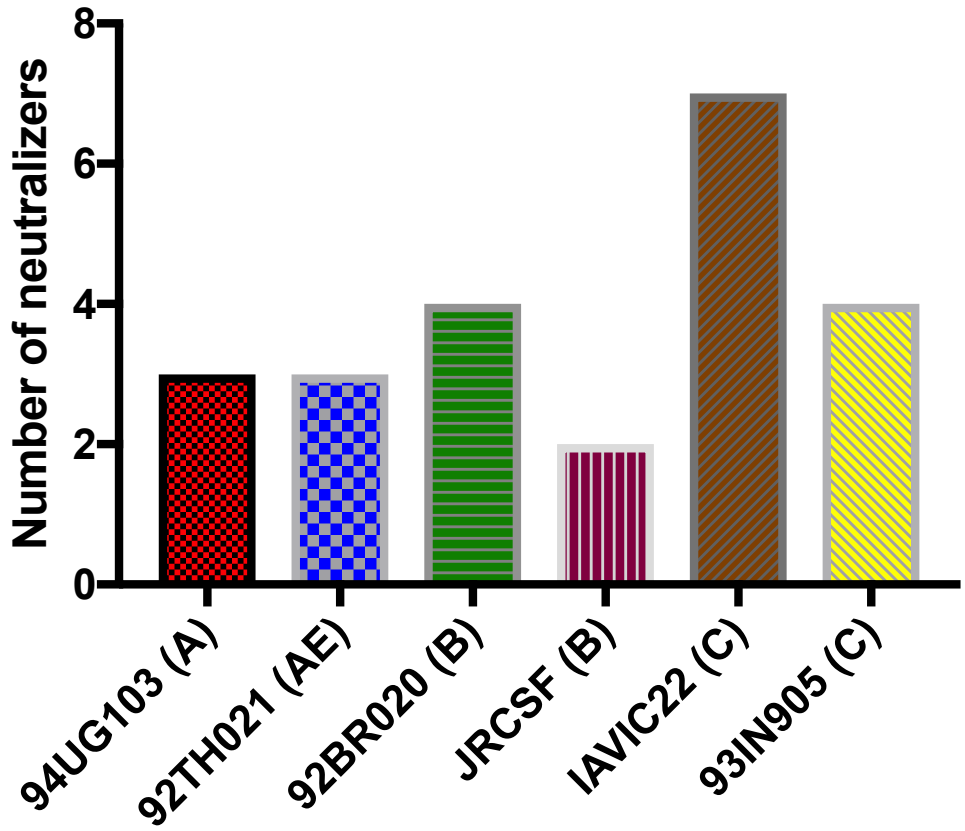


Figure 5:19: The proportion of individuals neutralising pseudoviruses from specific HIV clades. Plasma from HIV infected participants collected at 48 months PI was tested for neutralising ability on a 6-virus panel. HIV subtype C pseudoviruses were most commonly neutralised. The x-axis represents the pseudoviruses while the y-axis represents the number of neutralisers. GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to analyse the data.

5.5: Associations between Fab- and Fc-mediated antibody functions

Previous studies have suggested that there is a mechanistic link between the Fc and Fab of antibody functions as these are intrinsically linked within the GCs by mechanisms that use similar cytokines and enzymes. Germinal centres are important as antibodies undergo both class switching and somatic hypermutation that enhance Fc- and Fab-mediated functions, respectively in these environments.

Associations between all the Fc-mediated functions tested at the three time points with breadth development at 48 months PI were done. Only ADCD function at 3 months PI negatively correlated with breadth at 48 months PI, rho= -0.3175, p=0.0205 (Figure 5.20). This finding may suggest that robust complement activity in early HIV infection may reduce viremia, which the development of breadth relies upon, as suggested in other studies. Alternatively, high ADCD activity, which is mostly modulated by IgM, could suggest poor class switching which may translate to poor GC reaction. Thus, development of breadth would be delayed, or in part, inexistent. However, ADCD function did not negatively correlate with HIV viremia at any time point tested, which may suggest that another mechanism may be in play.

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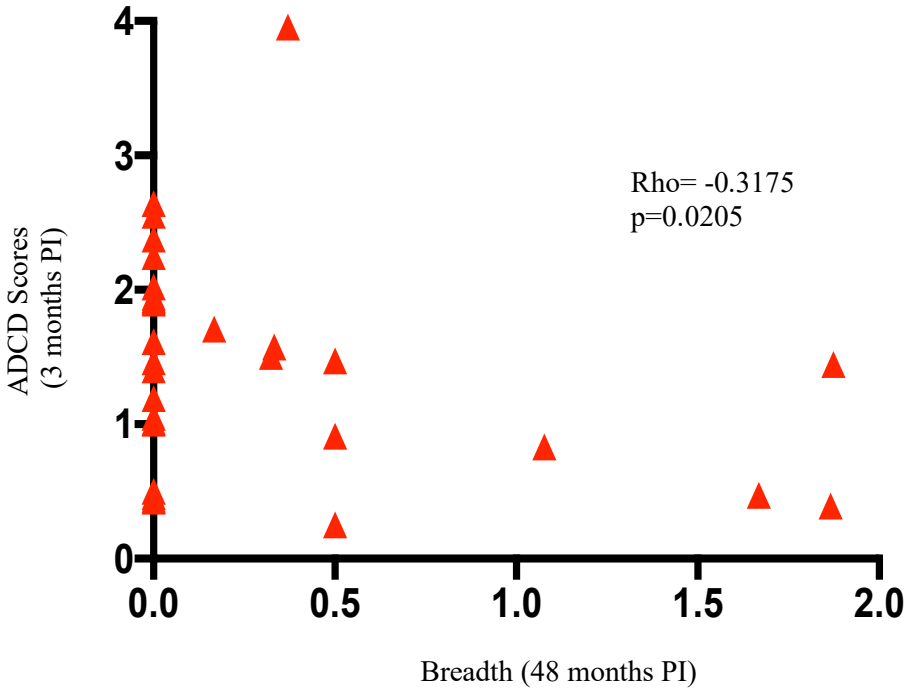

Figure 5:20: The correlation between ADCD and neutralisation breadth. Antibody-dependent complement deposition at 3 months post-HIV infection negatively correlated with neutralisation breadth at 48 months PI. Significance was defined by p <0.05. Test; non-parametric Spearman's correlation. PI; post-HIV infection.

5.5: Associations between Fab- and Fc-mediated antibody functions in individuals with any level of breadth

Since a large proportion of individuals showed no neutralising activity (breadth of > 0%), association analysis between Fc effector and Fab-mediated functions was restricted to individuals who showed any level of breadth, n=15. At both 3 months PI and 12 months PI, ADCP function positively correlated with breadth development at 48 months PI, rho=0.6963, p= 0.005 and rho=

0.6095, p= 0.018 respectively (Figure 5.21, A and B). This may suggest that quality ADCP function in early HIV infection may be a proxy of neutralisation breadth development.

On the other hand, HIV viral load measurements at 3 months PI negatively correlated with the neutralisation breadth at 48 months PI, rho= -0.6788, p= 0.0066 (Figure 5.21, C). This may imply that, in the setting of high viremia in early HIV infection, an HIV infected individual may fail to select for B cells that may further develop breadth, probably due to destruction of the immune system by HIV. All of the other parameters assessed in the neutralisers had no significant associations with the development of breadth.

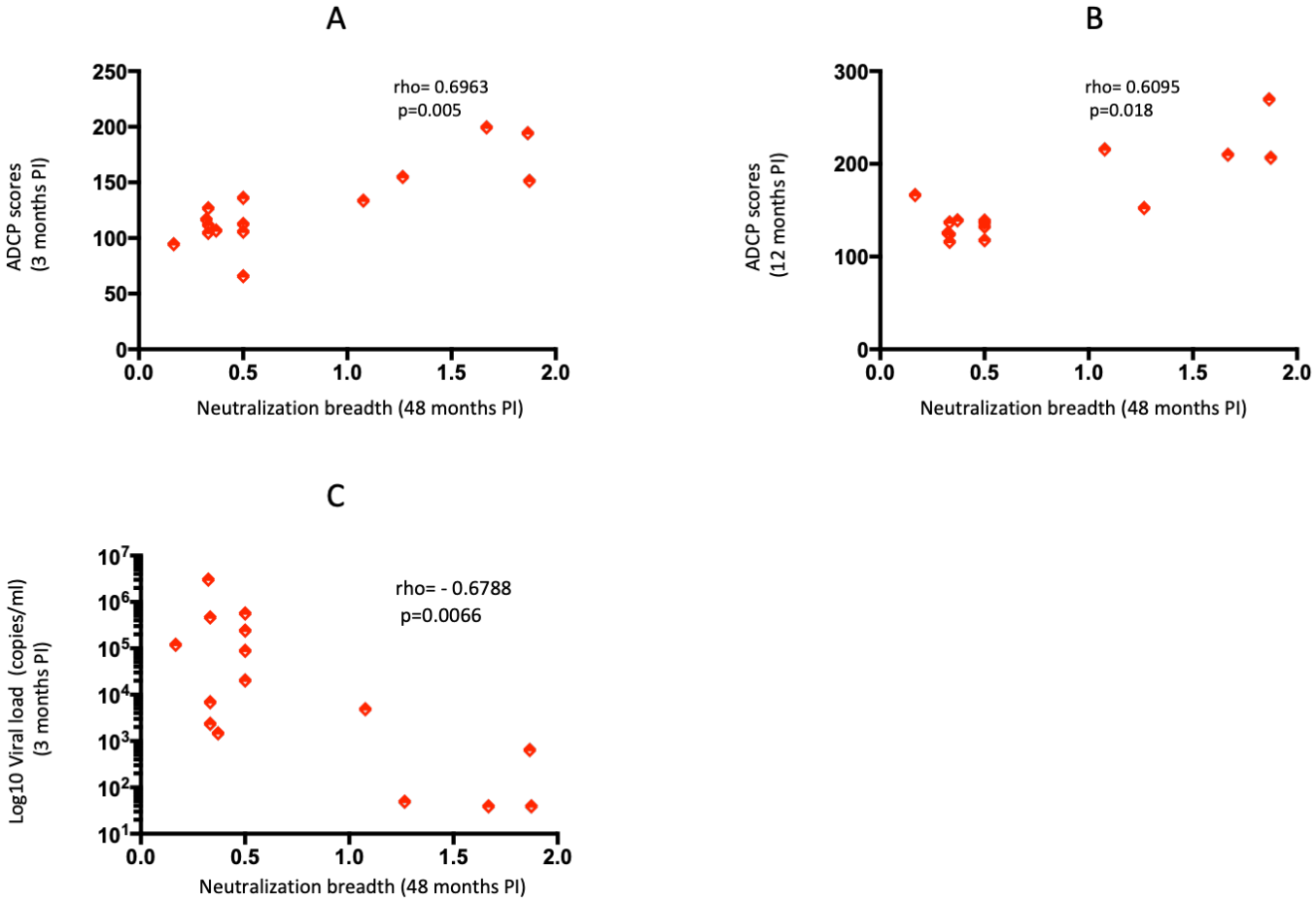

Figure 5:21: Associations between neutralisation breadth with ADCP and HIV viral load measurements. (A and B) Antibody-dependant cellular phagocytosis at 3- and 12 months PI positively correlated with neutralisation breadth at 48 months PI, respectively. (C) Contrariwise, the significant negative association between HIV viral load measurements at 3 months PI with neutralisation breadth, at 48 months PI was observed. Spearman's correlation rho values were defined by $p < 0.05$ for significance. PI; post-HIV infection. HIV viral load measurements are presented in log10.

5.6: Discussion

This study had the advantage of using antiretroviral treatment (ART) naïve plasma samples that were longitudinally sampled as early as 3 months PI. Similar to previous studies in adult cohorts, Fc-mediated antibody function in HIV infection was readily detectable in plasma 3 months PI [134, 334]. Furthermore, and consistent with the literature [134], the response levels of ADCD, ADNP and ADNK increased over time. However, despite earlier reports of a consistently high level of ADCP activity over time [134], this study shows that ADCP activity gradually increases after infection, peaking at 12 months PI, before a slight reduction at 48 months PI.

Other studies have also reported conflicting findings. For instance, it was reported that HIV-specific ADCP activity increases significantly in the course of HIV infection [334]. Other studies reported a decline in ADCP activity as HIV disease progressed [335, 336] and this decline was associated with either the levels of CD4+ T cell counts or HIV viral load or decreased Fc-receptor expression on innate immune cells. Antibody-dependent cellular cytotoxicity has also been shown to peak at 6 months and later on decay as HIV progressed [337]. This finding is probably backed up by the RV144 trial, where ADCC activity decayed [240].

Nevertheless, Baum and others reported an increasing ADCC titre over time in HIV infection, suggesting an increase of ADCC activity over time [143]. While we have differences in findings on the kinetics of Fc responses in HIV, it is clear that these responses are generated earlier on in infection. It is plausible that the differences in the kinetics may be due to the lack of a standardised assay in different laboratories.

Since the increase in activity of ADNP and ADNK could partly be explained by increasing titres of IgG subclasses [134, 143], it was not clear why ADCD function significantly increased over time since IgM mediates it. Previous work showed that IgM effectively activates complement due to its pentameric structure [338, 339]. Since it has well been documented that IgM is the first antibody to be detected following HIV infection [15], its increasing activity via ADCD function at 48 months PI is not apparent. However, it is feasible that due to the constant mutation and immune escape by HIV, any new mutant viruses during chronic HIV infection may be processed as a "new infection" at the GCs, prompting the generation of IgM antibodies that continuously mediate complement even during chronic infection.

Nevertheless, the increase in Fc activity was supported by the trend of increasing proportions of the number of individuals with high individual Fc function over time. The increase in Fc activity may suggest that the length of infection may play a role in driving better Fc function. Only ADCP at 3 months PI negatively correlated with viral load measurements at 3 months PI. Monocytes are targets of HIV infection [340], and they enhance viral replication in HIV and are dysregulated [341], and this may interfere with their recruitment by antibodies. Consistent with literature [342], data here shows that ADCP activity may play a role in viremia control.

It is thought that if different Fc effector functions offer a coordinated immune response, an HIV infected individual may better control viremia. For instance, elite controllers displayed a more functionally coordinated innate immune-recruiting response involving natural killer cell functions, monocyte and neutrophil phagocytosis, and complement [146]. Additionally, individuals who failed to generate bnAbs were shown to have significant discordance between Fc effector functions in early HIV infection, suggesting that concordance in Fc responses may be a signature for bnAbs

development [134]. This study showed significant correlations between Fc responses suggesting a functionally well-coordinated response. Furthermore, by calculating Fc polyfunctionality scores, Richardson and others showed that individuals with high Fc polyfunctionality seemed to have intact immune systems that would later lead to bnAbs development [134]. Moreover, Fc polyfunctionality has been implicated in the induction of multiple Fc functions simultaneously in elite controllers [146]. Similarly, high and well-coordinated Fc polyfunctionality responses targeting the V2 loop epitopes may have provided vaccine efficacy observed in RV144 and not VAX003 vaccine trial [343].

Of the 53 study participants, only 35.8% (19/53), 30.2% (16/53) and 41.5% (22/53) exhibited high Fc polyfunctionality at approximately 3 months, 12 months and 48 months PI respectively. While Fc polyfunctionality has not been studied in-depth, it has been directly linked with the development of breadth [134] and with elite controllers [146]. Since both bnAbs generation and the ability to be an elite controller is rare, this may explain why Fc polyfunctionality is rare, since all the three are closely associated.

Moreover, data shows that individuals who make these quality Fc-mediated responses in acute HIV tend to make them as the disease progresses. This may suggest that early priming of Fc responses may be vital in maintaining them in the course of HIV. Interestingly, similar to the increase of individual Fc responses over time, Fc polyfunctionality activity increased with time. While there was a slight drop in the proportions of those with high polyfunctionality at 12 months PI, these quality responses increased at 48 months PI, implying that length of infection plays a role. Indeed, a higher proportion of individuals, 15.1% (8/53), exhibited high polyfunctionality at 48 months PI.

Nevertheless, individuals with high Fc polyfunctionality had similar median viral loads to those with low polyfunctionality, indicating that viremia may not play a role in driving such robust responses. Indeed, Fc polyfunctionality at 3 months PI negatively correlated with viral load measurements at 12 months PI, suggesting that in the presence of high Fc polyfunctionality, viremia is more likely to be low. Nevertheless, individuals with low Fc polyfunctionality had significantly higher CD4+ T cell counts than those with high Fc polyfunctionality at 48 months PI, implying that HIV viremia is necessary for driving high Fc polyfunctionality. However, at the other two time points, participants with low Fc polyfunctionality did not preserve CD4+ T cell counts as compared to individuals exhibiting high Fc polyfunctionality. From the results, it was concluded that Fc polyfunctionality is rare and may be influenced by the length of HIV disease.

Similarly, data indicated that the evolution of bnAbs is rare. Indeed, only 10-20% of HIV infected individuals develop bnAbs, albeit after 2 – 4 years of infection [159, 313].
This study investigated the development of bnAbs responses against HIV approximately 48 months after HIV infection and showed similar results that the development of breadth was rare as previous studies [159, 313].

Interestingly, subtype C pseudoviruses were more readily neutralised despite most participants having been infected with HIV subtype A. While this could be as a result of the cross-reactive nature of bnAbs, HIV subtype C virus has been associated with the development of bnAbs [159], and that, may in part, explain its neutralisation. However, since not everyone infected with subtype C virus develops bnAbs, there must be another explanation for this.

Unlike previous studies [313, 344, 345], no association was observed between antibody neutralisation function and viral load or CD4 counts. Only a small number of the cohort participants generated any neutralisation breadth in the follow-up period. It is therefore highly likely that the small sample size negated the possibility of performing any meaningful statistical associations.

Notably, none of the Fc-mediated functions tested had significant associations with breadth except ADCD function. This may infer that quality complement activity 3 months post PI may reduce viremia in early HIV infection, thus eliminating one of the crucial drivers of bnAbs development [159]. Although the lack of strong associations between bnAbs and Fc-mediated responses, trends of positive associations were evident and probably with a larger cohort with more individuals generating neutralisation breadth significance may be attained. This further emphasizes that both Fc and Fab are intrinsically linked and modulated distinctly in the GCs reaction, similar to previous suggestions [134].

However, when only the subset of participants who developed any level of neutralisation was considered, and associated with CD4+ T cell counts, viral load measurements and Fc-mediated functions, ADCP function at 3- and 12- months PI significantly predicted the development of breadth. This strongly suggests that the quality ADCP function in early HIV infection may be a driver of the development of bnAbs, most likely through the preservation of CD4 cells through control of viremia. Indeed, ADCP at 3 months PI negatively correlated with viral load measurements at 3 months PI implicating its role in the control of viremia in the whole cohort (Individuals with and without neutralisation breadth).

In summary, this chapter reports:

- Fc-mediated functions were readily detected in all individuals as early as 3 months PI, unlike Fab-mediated function, which was only detected in a few individuals and late in HIV infection.
- ADCP function at 3- and 12- months PI significantly predicted the development of breadth, implying that early ADCP activity may be a driver or a signature of bnAbs development.
- Fc polyfunctionality in early HIV infection negatively correlated with viral load measurements, suggesting a role of Fc polyfunctionality in controlling HIV viremia.

Nevertheless, more studies will be required to understand the link between Fc and Fab functions in order to harness these responses in a vaccine strategy.

Chapter 6 Early immunological events associated with antibody function quality

6.1: Literature review

Despite the human immunodeficiency virus (HIV) -specific Fab- and Fc-mediated antibody functions being mostly studied in HIV infected adults, little is known about the cellular mechanisms involved in the generation of these antibody functions. Generally, it is accepted that most current vaccines rely on long-lasting protective antibodies that are present in serum or on the mucosa [346], and eliciting such an antibody-based HIV vaccine may confer protection. T follicular helper (Tfh) cells are CD4+ T cells specialised in providing help to B cells to enable B cell differentiation into affinity-matured long-lived plasma cells and memory B cells. Tfh cells are critical for affinity-maturation, a process where antigen-specific B cells undergo several rounds of somatic hypermutation in the germinal centres (GCs) to evolve high-affinity somatically mutated B cell receptors (BCRs) [75]. Additionally, Tfh cells are critical for antibody class switching [174], a process that enables antibodies to trigger various Fc-mediated activities depending on their isotypes [146, 347-349].

Due to this critical role of both Tfh and B cells in the generation of functional antibodies, understanding their interactions in early HIV infection remains essential. Indeed, Tfh cells have been linked with the generation of broadly neutralising antibodies (bnAbs) in HIV infection [225, 350], and antibody quantities may play a role in determining functionality. In HIV infection, high overall plasma immunoglobulin (Ig) G levels and anti- Envelope (Env) IgG binding titres were associated with the development of breadth in a large International Acquired Immunodeficiency Syndrome (AIDS) Vaccine Initiative (IAVI) protocol C cohort [159], suggesting an increased GCs activity may favour both. While both Fab and Fc regions of the antibody are encoded by different genes [195], recent findings show that they may be jointly regulated as they are mechanistically

linked in the GCs [134]. Perhaps more convincing is that both class-switch recombination and somatic hypermutation require the enzyme activation-induced cytidine deaminase to enable transcription and deoxyribonucleic acid (DNA) cleavage, respectively [351].

Moreover, since the constant region can slightly affect the antibody's Fab function [352, 353], both Fab and Fc regions could be dependent on each other. As GCs are not easily accessible in human studies due to surgical and ethical restrictions, the chemokines CXCL13 and B cell activating factor (BAFF) in plasma may present a good proxy of GCs activity and can be evaluated to inform the interaction between Tfh and B cells in the GCs [96, 212, 213]. This helps create a better understanding of critical GCs immunological processes in disease.

In this objective, I explored the association between the frequencies of general and HIV-specific B and T cells and the downstream antibody levels and functions. Also, I investigated whether the chemokines BAFF and CXCL13, proxies of GCs activities, were associated to the observed T and B cell frequencies early in infection. Both HIV-specific and general T and B cell subsets were associated with CXCL13 and BAFF plasma levels, HIV-specific Fab- and Fc-mediated antibody functions and HIV-specific antibody levels.

As numerous antigens had been used to determine HIV-specific antibody levels in the Luminex assay, I used data from the BG505 SOSIP clade A immunogen as the representative antigen as antibody levels to the different antigens showed similar trends (Chapter 4, subsection 3.1). Data from B and T cell phenotypes, BAFF and CXCL13 levels that were determined during early HIV infection (3 months post HIV-infection-PI) were associated with HIV-specific IgG, IgA1, IgA2 and IgM levels and subclasses $IgG_{1,}$ IgG_2, IgG_3 and IgG_4 and non-neutralising function determined

at 3-, 12- and 48-months PI and antibody neutralisation breadth determined at 48 months PI, as described in the previous chapters.

Previous studies have associated general Tfh cell subset frequencies with the quality of antibody responses; or where antigen-specific subsets were determined, a cross-sectional approach was used. The current study offers additional important information to the field by associating HIV-specific T and B cell subsets and downstream antibody function. It is likely that cellular events much earlier in the disease maybe more reflective of observed antibody properties and functions at a particular time point. Using a longitudinal study is therefore more informative.

I, therefore, hypothesised that the frequencies of HIV-specific Tfh and B cells in acute/early HIV infection would be good predictors of the quality of antibody responses that are observed downstream during chronic HIV disease.

6.2: Objective

To establish the association of early T and B cell subset frequencies with GCs activities, Fab- and Fc-mediated antibody functions and antibody levels in chronic HIV infection.

6.3: Results

6.3.1: Associations of general and HIV-specific CD4+ T cell phenotypes early in HIV infection with downstream GCs activities, antibody levels and functions

Antibody levels and functions were associated with general CD4+ T cell frequencies. Fc-mediated functions increased with higher general CD4+ T cell frequencies, while HIV-specific antibodies decreased. Only IgG_1 levels at 48 months post-HIV infection (PI) showed significant decrease with

increasing general CD4+ T cell frequencies with (Table 6.1). Antigen experienced CD4+ T cells, defined as CD27+ CCR7+ CD4+ were then considered, this subset positively associated with IgG and IgA_2 levels at 48 months PI and negatively associated with antibody-dependent natural killer cell function, marked by secretion (ADNK - IFN-γ) at 48 months PI and by the expression of macrophage inflammatory protein-1α (ADNK - MIP-1α) at 3 months PI.

Association of the subset CD45RA- CXCR5+ CD4+, which is representative of the memory Tfh cells which are more specific for offering B cell help was then considered. A significant positive association was observed with IgG antibody levels at 3 months PI, IgG and IgG_1 antibody levels at 12 months PI and with IgA and IgG_1 antibody levels at 48 months PI. Within the memory Tfh cells, PD-1+ CXCR3- CXCR5+ CD4+ cells are representative of activated Tfh cells, which have been reported to be associated with the development of breadth in HIV infection [225]. This subset of cells was found to be associated with antibody-dependent neutrophil phagocytosis (ADNP), IgG and IgG_1 levels at 3 months PI and negatively with IgG_2 and IgG_4 levels at 12 months PI. The subset ICOS+ PD-1+ CXCR5+ CD4+, which is representative of activated Tfh cells, was also found to be associated with IgG levels at 3 months PI and with IgM levels at 48 months PI.

When HIV-specific Tfh cells were considered, an increase in frequency was observed with higher antibody levels and Fc mediated functions and a significant association was observed for the Fc-mediated function, ADNK - IFN-γ release at 3 months PI. Germinal centre activities marked by BAFF and CXCL13 levels showed no correlation with any of the CD4+ T cell subsets (Table 6.1).

Table 6.1: Associations between CD4+ T cell subsets at 3 months PI with HIV-specific antibody levels and functions at 3-,12- and 48 months PI.

CD4+ subsets measured at 3 months post-infection	**Antibody subclass or function showing association to CD4+ T cell subset**	**Time post-infection when the association was observed (months PI)**	**Spearman's rho value**	**p-value**
General CD4+	IgG_1	48	-0.3399	0.0147
CD27+ CCR7+ CD4+ (Central memory T cells)	IFN-γ	48	-0.2885	0.0401
	$ADNK_{MIP\text{-}1\alpha}$	3	-0.2882	0.0403
	IgA2	48	0.3407	0.0144
	IgG	48	0.365	0.0084
CD45RA- CXCR5+ CD4+ (Memory Tfh cells)	IgA	48	0.2768	0.0493
	IgG	3	0.3115	0.0310
	IgG	12	0.3210	0.0216
	IgG_1	12	0.3073	0.0283
	IgG_1	48	0.3100	0.0268
PD-1+ CXCR3- CXCR5+ CD4+ (Activated Tfh cell subset)	ADNP	3	0.2900	0.0380
	IgG	3	0.3034	0.0304
	IgG_2	12	-0.2959	0.0350
	IgG_4	12	-0.3067	0.0286
ICOS+ PD-1+ CXCR5+ CD4+ (Activated Tfh cell subset)	IgM	48	0.3207	0.0230
	IgG	3	0.3135	0.0251
OX40+ CD25+ CXCR5+ CD4+ (HIV-specific Tfh cells)	$ADNK_{IFN\text{-}\gamma}$	3	0.3314	0.0175

T cell subsets at 3 months post-HIV infection (PI) were associated with HIV-specific antibody levels and functions at 3-, 12- and 48 months PI. Spearman's rho values and their associated p-values are shown in the cells. Only associations with p values <0.05 are presented. PI – post HIV infection. ADNP - antibody-dependent neutrophil phagocytosis; $ADNK_{IFN\text{-}\gamma}$ - antibody-dependent natural killer cell (ADNK) - *interferon gamma secretion;* $ADNK_{MIP\text{-}1\alpha}$, MIP-1α - macrophage inflammatory protein 1-alpha. Test; Spearman's correlation.

6.3.2: Associations of general and HIV-specific B cell phenotypes early in HIV infection with downstream antibody levels and functions

Apart from dysregulating the T cell compartment, HIV infection has also been shown to cause dysregulation and dysfunction of other lymphocyte populations, including B cell subsets [354, 355]. To understand how these changes in the B cell compartment may influence downstream antibody levels and functions, B cell subsets in early HIV infection (3 months PI) were associated with GCs activities (based on the B cell activating factor (BAFF)) and CXCL13 levels as proxies) and antibody levels and functions at 3-, 12- and 48 months PI.

Associations of mature B cell (CD10- CD19+) frequencies with BAFF and CXCL13 as proxies of GC activity and with antibody functions showed an increase in B cells subsets with increasing antibody function with only the antibody-dependent cellular phagocytosis (ADCP) and IFN-γ functions at 3 months PI (Table 6.2) being significantly associated.

When the antigen-experienced class-switched B cells (CD10- CD19+ CD20+ IgG+) were considered, higher frequencies were observed with reducing Fc-mediated antibody function. The negative association was significant for ADCP function at 48 months PI. A positive association of atypical B cells (CD10- CD19+ CD21- CD27-), which proliferate poorly in response to B cell stimuli [356], with IgA_1 levels was observed at 12 months PI and with IgG_2 levels at 48 months PI. Naïve B cells (CD10- CD19+ CD21+ CD27-) were negatively associated with the levels of IgM, IgG_1 and IgG_3 at 3 months PI, IgG_3 at 12 months PI and IgG_4 at 48 months PI.

Activated B cells (CD10- CD19+ CD21- CD27+), which are the subset that migrates into the B cell follicles to undergo proliferation and selection and thereby exit as memory B cells or plasma

cells [218], showed positive correlations with CXCL13, IgG_1, IgG_2 and IgG_3 levels at 3 months PI, IgG levels at 12 months PI and IgG_4 levels at 3- and 12-months PI.

Resting memory B cells (CD10- CD19+ CD21+ CD27+), a subset that is often decreased in HIV, showed inverse associations with antibody-dependent neutrophil phagocytosis (ADNP) at 3 months PI, antibody-dependent complement deposition (ADCD) at 12- and 48-months PI and CXCL13 and IgG_2 levels at 48 months PI.

HIV-specific B cells (CD10- CD19+ CD20+ IgG+ gp120+), the cells that would be responsible for HIV-specific antibody generation, were considered, direct associations with IgA_1, IgG, IgG_2, IgG_3, levels at 3 months PI and IgG_3 levels at 48 months PI were observed. When their association with antibody function was considered, participants with higher frequencies tended to have lower Fc mediated functions although this did not reach significance.

Table 6.2: Associations between various B cell subsets at 3 months PI with GC activities, antibody levels and functions at 3-,12- and 48 months PI.

B cell phenotypes determine early in infection (3 months)	Antibody subclass or function showing association to B cell subsets	Time post-infection when the association is observed (months PI)	Spearman's rho value	p-value
CD10- CD19+ (Mature B cells)	ADCP	3	0.4316	0.0013
	$ADNK_{IFN-\gamma}$	3	0.2865	0.0375
CD10- CD19+ CD20+ IgG+ (Class switched B cells)	ADCP	48	-0.2854	0.0383
CD10- CD19+ CD21- CD27- (Atypical B cells)	IgA1	12	0.2727	0.0482
	IgG_2	48	0.2952	0.0319
CD10- CD19+ CD21+ CD27- (Naïve B cells)	IgM	3	-0.3685	0.0066
	IgG_1	3	-0.2952	0.0319
	IgG_3	3, 12 *	-0.271, -0.3105	0.0496, 0.021
	IgG_4	48	-0.3114	0.0232
CD10- CD19+ CD21- CD27+ (Activated B cells)	CXCL13	3	0.38	0.0062
	IgM	3, 12, 48 *	0.43, 0.3448, 0.3064	0.0012, 0.0114, 0.0257
	IgG	12	0.4063	0.0025
	IgG_1	3	0.4285	0.0014
	IgG_2	3	0.2867	0.0374
	IgG_3	3	0.3132	0.0224
	IgG_4	3, 48 *	0.3261, 0.2892	0.0172, 0.0357
CD10- CD19+ CD21+ CD27+ (Resting B cells)	CXCL13	48	-0.3924	0.0037
	ADCD	12	-0.3442	0.0116
	ADCD	48	-0.3818	0.0136
	ADNP	3	-0.3918	0.0037
	IgG_2	48	-0.3403	0.0127
CD10- CD19+ CD20+ IgG+ gp120++ (HIV-specific B cells)	IgA1	3	0.3642	0.0073
	IgG	3	0.2919	0.0339
	IgG_2	3	0.3095	0.0241
	IgG_3	3, 48	0.4014, 0.3	0.0029, 0.0291

B cell subsets at 3 months post-HIV infection (PI) were associated with GCs activity, antibody levels and functions at 3-,12- and 48 months PI. Spearman's rho values and their associated p-values are shown. Only associations with p-values <0.05 are presented. PI – post-HIV infection. ADCP - antibody-dependent cellular phagocytosis; ADNP - antibody-dependent neutrophil phagocytosis; ADCD - antibody-dependent complement deposition; $ADNK_{IFN-\gamma}$ - antibody-dependent natural killer cell (ADNK) - *interferon gamma secretion*; * represents where more than one time point was compared and the resulting Spearman rho value or p value, for each time point shown respectively. Test; Spearman's correlation.

6.3.3: Associations between early B cell cytokines levels (3 months PI) with downstream HIV-specific antibody quantities and functions in chronic HIV infection

Previously, CXCL13 and BAFF have been shown to be associated with GCs activity [96, 212, 213]. In this section, I sought to establish if CXCL13 and BAFF levels determined early in the infection (3 months PI) were associated with antibody levels and functions at 3-,12- and 48 months PI. This would be an indicator of whether early GCs quality influences antibody responses during chronic HIV infection.

There was no correlation between BAFF levels with antibody levels and functions at 3-,12- and 48 months PI. However, direct correlations were observed for CXCL13 levels with IgA_1 and IgG_3 levels at 3 months PI, IgM levels at 12 months PI and IgA_2, IgG, IgG_1 and IgG_2 levels at 3-, 12- and 48 months PI (Table 6.3). It appears that CXCL13 was a better predictor of downstream antibody levels than was the B cell-activating factor, BAFF.

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Table 6.3: Associations between CXCL13 levels at 3 months PI with various immune correlates of protection at 3-,12- and 48 months PI.

Soluble factor measured at 3 months PI	Antibody levels and functions associated with the soluble factors measured	The time when the association is seen (months PI)	Spearman's rho value	p-value
CXCL13	$ADNK_{CD107\alpha}$	3	-0.3138	0.0221
	IgA2	3	0.4212	0.0017
	IgA2	12	0.3397	0.0128
	IgA2	48	0.278	0.0438
	IgA1	3	0.33	0.0175
	IgM	3	0.49	0.0002
	IgM	12	0.5217	<0.0001
	IgG	3	0.5459	<0.0001
	IgG	12	0.3874	0.0042
	IgG	48	0.3706	0.0063
	IgG_1	3	0.5641	<0.0001
	IgG_1	12	0.3759	0.0055
	IgG_1	48	0.3315	0.0153
	IgG_2	3	0.3861	0.0043
	IgG_2	12	0.3681	0.0067
	IgG_2	48	0.3449	0.0114
	IgG_3	3	0.4912	0.0002

CXCL13 levels at 3 months PI was associated with antibody levels and functions at 3-,12- and 48 months PI. Spearman's rho values and their associated p-values are shown. Only associations with p-values <0.05 are presented. PI – post-HIV infection. $ADNK_{CD107\alpha}$ - antibody-dependent natural killer cell (ADNK) CD107α expression- Test; Spearman's correlation.

6.3.4: Summary matrix associating immunological parameters measured in the course of HIV infection: a systems approach

Significant associations were shown in tables 6.1, 6.2 and 6.3. However, in order to visualise all possible associations (both significant and non-significant) between cellular subsets, B cell cytokine levels, HIV-specific antibody titres and both HIV-specific Fab- and Fc-mediated antibody functions in HIV infection, a correlation matrix was used. In brief, the Spearman's correlation values for each association and their p-values were determined. Using the R package *corrplot*, on RStudio 1.0.143 (PBC, Boston, MA), the associations were visualised. In order to regroup the variables according to the level of association as described by the Spearman's rho values, the correlation matrix was reordered using the "*hclust"* algorithm on R. (Figure 6.1). The symbol "x" was used to mark out all insignificant associations ($p<0.05$). This unbiased systems approach summarises the associations described earlier on in the chapter.

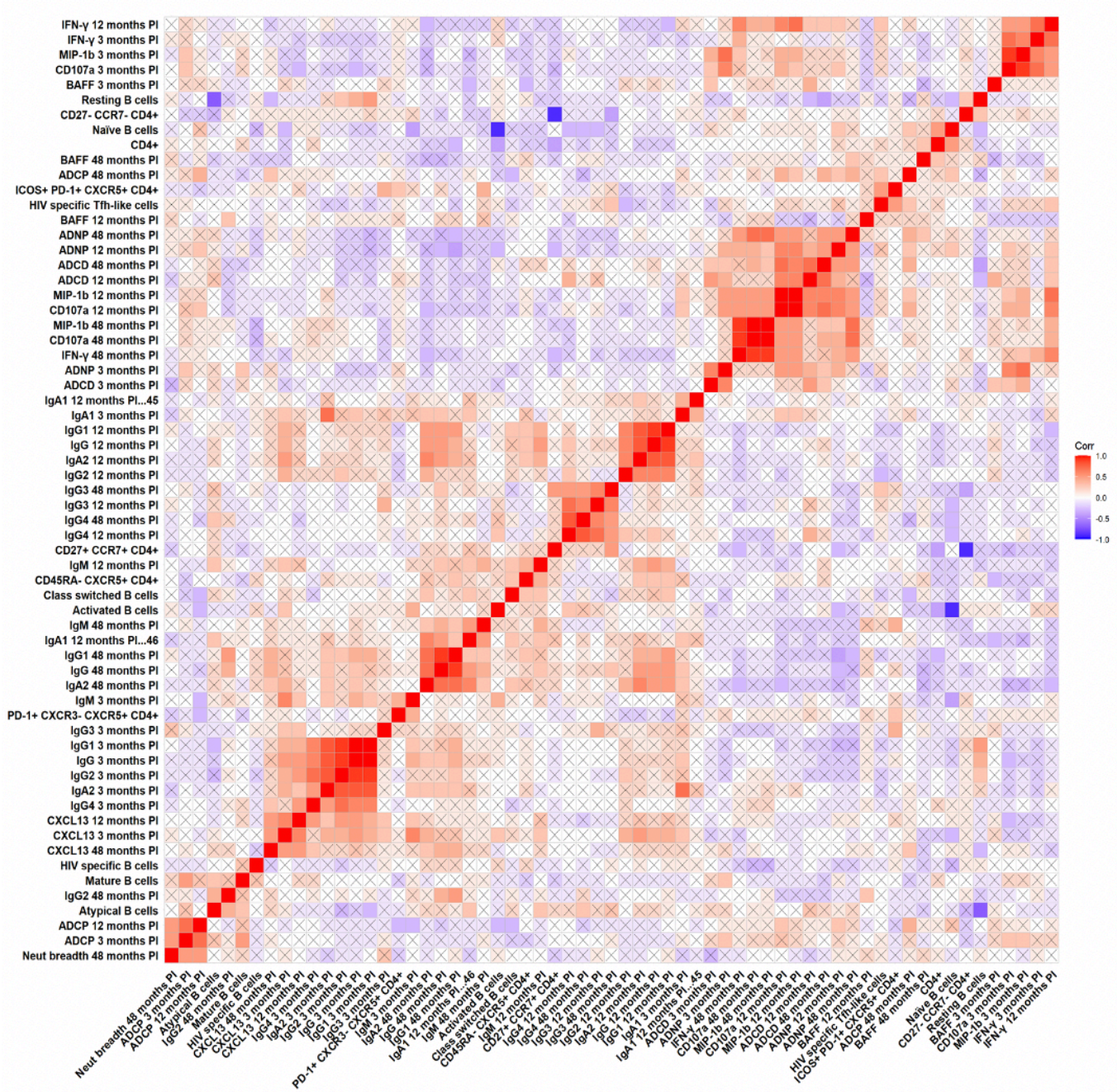


Figure 6:1: Heatmap depicting the hierarchical clustering of the correlation matrix showing the associations between all immunological variables tested during the first 48 months post-HIV infection. As per the key provided on the right side of the figure, red colour indicates significant strong positive correlations (rho >0.7), peach colour represents moderate positive correlations (0.4 < rho < 0.7), while white represents weak positive correlations (0 < r <0.4). The inverse correlation is represented by the purple colour that indicates a moderate negative correlation (-0.5 < rho < 0), while blue depicts a strong negative correlation (-1 < rho < 0.5). Both axes represent immunological variables tested during the first 48 months post-HIV infection. The symbol "x" was used to mark out all insignificant associations in the matrix where p>0.05. PI; post-HIV infection. Figure was generated on RStudio 1.0.143 (PBC, Boston, MA).

6.4: Discussion

In this chapter, I assessed the association of early general and HIV-specific T and B cell subsets with GCs activity, HIV-specific antibody levels and functions. The general CD4+ T cells frequencies were negatively associated with IgG_1 levels at 48 months PI. This could suggest that, in the setting of high viremia that is required for robust anti-HIV antibody responses [294], CD4+ T cells would be depleted as earlier described [22]. Similarly, CD45RA- CXCR5+ CD4+ T cells negatively associated with $ADNK_{IFN\text{-}\gamma}$ function and CXCL13 levels further emphasizing the role of HIV viremia in driving GCs activity. This could mean that while high viremia may induce quality GCs function leading to higher CXCL13 levels [294], this would confer quality Fc-mediated function ($ADNK_{IFN\text{-}\gamma}$ function) since intrinsic immune factors within the GCs are mechanistically linked [134].

Although not HIV-specific, CD45RA- CXCR5+ CD4+ T cells were associated with HIV-specific antibody levels. This is in line with previous observations [99, 225], where general (non HIV-specific) Tfh cells have been reported to offer B cells help, which may lead to high titres of functional antibodies. The Tfh subset PD-1+ CXCR3- CXCR5+ CD4+, which is highly functional and has been shown to correlate with the development of bnAbs in HIV infection, was directly associated with ADNP function. This supports the mechanistic link of intrinsic immune factors (Fc and Fab functions) within the GCs. Furthermore, PD-1+ CXCR3- CXCR5+ CD4+ T cells were positively associated with IgG and IgG_1 levels but not IgG_2 and IgG_4. Since PD-1+ CXCR3- CXCR5+ CD4+ T cells have been linked with the generation of bnAbs in HIV, it is plausible that it is responsible for driving IgG_1 and IgG responses. Indeed, superior IgG_3/IgG_1 responses have been reported in elite controllers who demonstrated Fc polyfunctionality [146]. Thus, this finding

may imply that PD-1+ CXCR3- CXCR5+ CD4+ T cells that have been shown to predict the development of bnAbs, may also play a role in enhancing robust IgG_3/IgG_1 responses.

Likewise, ICOS+ PD-1+ CXCR5+ CD4+ T cells, had a direct association with IgM and IgG levels, suggesting its role in driving antibody generation. Indeed, ICOS+ CXCR5+ CD4+ T cell frequencies have been linked with the production of hepatitis C virus-specific antibodies [357], while $CXCR5^+$ $PD\text{-}1^+$ $CD4^+$ T cells are critical for the development of bnAbs in HIV infected subjects [99]. Additionally, the frequencies of CD4+ CXCR5+ PD-1+ Tfh cells have been shown to negatively associate with CD4+ T cell counts in HIV infected individuals [358], implying that high viremia that drives low CD4+ T cell counts, is vital in driving high antibody levels through enhanced GCs activity.

The alteration in the CD4+ T cell compartment due to HIV direct and indirect effects [220] also influenced other antibody responses. For instance, CD27+ CCR7+ CD4+ T cells negatively correlated with $ADNK_{IFN\text{-}\gamma}$ and $ADNK_{MIP\text{-}1\alpha}$ activities but were positively associated with IgA_2 and IgG levels. This may suggest that the preservation of CD27+ CCR7+ CD4+ T cells may be necessary for higher antibody titres.

The direct correlation between the frequencies of mature B cells with $ADNK_{IFN\text{-}\gamma}$ and ADCP function suggests that a larger repertoire of B cells influences the pool from which HIV antigen-specific B cells are selected from, and thus enhancing Fc-mediated functions. However, it is not clear why the class-switched B cells negatively correlated with ADCP function, since, antibody class switching enhances Fc-mediated functions depending on the isotypes [146, 347-349]. Nevertheless, since HIV disrupts the B cell compartment [354, 355], different B cell subsets had

dissimilar associations with antibody levels and functions. For instance, activated B cells had positive associations with antibody levels. This is consistent with reports that activated B cells initiate the GCs reaction, before exiting the B cell follicles as memory B cells or plasma cells to generate antibodies [119]. Moreover, activation of B cells in the first year of HIV infection has been reported to correlate with bnAbs development in chronic disease [99], highlighting the role of activated B cells in HIV disease.

Resting memory and naïve B cells were negatively associated with antibody levels suggesting that HIV viremia may drive the activation of resting memory and naive B cells resulting in a decrease of their frequencies with higher viremia. Furthermore, resting B cells were negatively associated with CXCL13 levels, highlighting the role of viremia in activating B cells and driving GCs activities.

Atypical B cells were positively associated with HIV-specific antibodies. HIV responses may be primarily from these population of cells ($CD21^{lo}/CD27^{-}$ B cells), which are expanded during chronic infection. However atypical B cells are known to putatively express the inhibitory receptor FCRL4 [359], which has decreased responses to B cell stimuli [360]. It is therefore possible that the relationship is not causal and may be reflective on an environment with high viremia, which is associated with high antibody levels and increased proportion of atypical B cells. Of importance is that HIV-specific B cells showed direct correlations with HIV-specific antibody levels emphasizing the benefit of antigen-specific phenotypes in understanding antibody function.

In summary, I have shown associations between early immunological events with antibody responses, functions and GCs activities that develop later in HIV infection. Higher frequencies of general T and B cell subsets were associating with higher HIV antibody levels and showed poorer

association to antibody functions. When BAFF and CXCL13 levels were used as a proxy for germinal centre reactions, CXCL13 was better associated with downstream antibody levels than BAFF. Similar to the phenotypic analysis, poor associations to antibody function were observed. It appears that CXCL13 is a better predictor of downstream antibody levels as compared to BAFF.

Several studies have addressed the relationship between early and late general T and B cell subsets with antibody quality in chronic infection. However, aside from evaluating general T and B cell subsets that are not antigen-specific, majority of the studies were cross-sectional and thereby reporting immunological events at a single time point. Moreover, these studies only considered downstream neutralising antibodies functions in HIV.

For instance, despite being a longitudinal study, Cohen and others associated only the general T cell subsets in early HIV infection with downstream bnAbs development in chronic HIV infection [99]. Higher frequencies of CXCR5+ PD-1+ CD4+ T cells in early HIV infection correlated with the breadth of plasma neutralising antibody responses in chronic HIV disease. Similarly, Locci and others used a longitudinal cohort to assess the association between general T cell subsets with the development of breadth. The frequencies of circulating memory PD-1+ CXCR5+ CD4+ T cells correlated with the development of bnAbs [225]. While both the Locci and Cohen studies used general T cell subsets in longitudinal cohorts, other studies have used general T cell subsets in cross-sectional cohorts.

For example, Lindquist and colleagues used lymph node samples to characterize Tfh cells in ART naïve and ART-treated HIV positive individuals [93]. They reported a significant expansion of Tfh cells in chronic HIV that was driving a perturbation of B cells differentiation leading to poor antibody function [93]. Likewise, Ranasinghe and colleagues reported a correlation of Gag-

specific CD4+ T cells with antibody breadth in ART naïve HIV controllers [100]. Similarly, CXCR3+ CXCR5+ PD-1^{lo} CD4+ T cells were relatively associated with the breadth of neutralising antibody responses in HIV controllers [361].

While these studies conclusively linked T cell subsets to bnAbs development, describing HIV-specific cell subsets and interactions, maybe a better correlate to antibody function, as these are the cells that specifically drive the HIV-specific responses. Also, analysing antigen-specific cell subsets in early infection may be a better predictor of subsequent antibody function in chronic HIV, since antibody function is often reflective of cellular events that occurred much earlier in an infection. Furthermore, previous studies have also correlated cellular events to neutralising antibody function and not addressing their role in non-neutralising functions. This is despite non-neutralising function being the only function that was associated with protection in the one vaccine clinical trial that showed modest efficacy [240].

The current study used an ART naïve longitudinal cohort of HIV infected individuals. Additionally, this study used HIV-specific subsets of B and T cells to describe HIV-specific antibody levels and functions later on in infection. While lymph node samples were unavailable, the pairing of peripheral antigen-specific cellular subsets with CXCL13 and BAFF provides useful information about GCs activities [96]. Additionally, associating HIV-specific cellular subsets with Fc-mediated functions is novel, since most studies have associated cellular responses with Fab-mediated antibody functions and not Fc-mediated functions. Therefore, this study offers insights in a systems biology approach that is antigen-specific, of the interaction between HIV and the human immune system without ART, and the resulting outcomes.

While HIV-specific Tfh cells had trends of association with HIV-specific Fc- and Fab-mediated functions, HIV-specific B cells had direct correlations with HIV-specific antibody levels. In principle, these findings may suggest that both HIV-specific Tfh and B cell subsets may influence these functions and levels. The modest sample size limited the analysis and significance. It is worthwhile exploring the HIV-specific responses in larger ART naïve longitudinal cohorts.

Chapter 7 Summary and conclusions

The effect of the human immunodeficiency virus (HIV) on the T and B cell compartments and its impact on germinal centres (GCs) activities has been well characterised [119, 218, 268, 354, 362, 363]. Specifically, HIV depletes CD4+ T cells [22] and alters the B cell compartment by increasing tissue-like memory B cells, activated B cells, plasmablasts and decreasing of the resting memory B cells [119, 217, 218, 354]. Similarly, associations between T and B cell subsets with GCs activities, HIV-specific antibody levels and functions have been described in HIV infection [93, 99, 100, 217, 225, 361]. However, these studies analysed general populations without demonstrating the contribution of HIV-specific subsets and function to downstream antibody responses. Additionally, some of the studies were cross-sectional, thereby reporting immunological events at single time points. Moreover, only antibody neutralising function has been previously considered [93, 99, 100, 225], with the association to Fc-mediated functions in HIV not included, despite their potential role in antibody based vaccine development. This is despite, non- neutralising activity being the only function that so far, has shown an association with protection in any HIV vaccine clinical trial, albeit being modest efficacy [154].

The current study analysed HIV-specific cell subsets which may provide the true reflection of the association with antibody responses since these are the cells that drive the HIV-specific responses. In addition, I determined the Fab- and Fc-mediated antibody functions. Thus, the current project improves our understanding in the field by using an antiretroviral treatment (ART) naïve longitudinally followed cohort to describe HIV-specific T and B cell subsets and to associate these with HIV-specific Fc- and Fab-mediated antibody functions, HIV-specific antibody levels and GCs activities further downstream the infection.

In this study, peripheral blood circulating T and B cell phenotypes and B cell cytokines levels (CXCL13 and BAFF) were determined in early HIV infection (3 months post-HIV infection (PI)) and associated with GCs activity, antibody quantities and qualities at 3-,12- and 48 months PI to determine cellular subsets that are predictive of quality antibody responses in chronic HIV infection, while the overriding aim was to determine how these interactions and functions impact on disease outcomes. Therefore, peripheral blood circulating T and B cell phenotypes, B cell cytokines levels, antibody quantities and qualities were associated with the markers of HIV disease progression at 3-,12- and 48 months PI. Longitudinal viral load measurements and CD4+ T cell counts were used as proxies for disease progression and outcome.

Therefore, the overall goal of this study was to understand the early cellular mechanisms involved in the generation of functionally relevant antibodies in chronic HIV infection. In particular, I aimed to:

1. To characterize peripheral blood HIV-specific T and B cell subsets during early HIV infection.
2. To determine HIV-specific antibody levels, isotypes and subclasses during HIV infection and as a proxy of germinal centre activity, determine the plasma levels of BAFF and CXCL13 cytokines before and during HIV infection.
3. To evaluate the quality of Fab and Fc-mediated antibody functions in the course of HIV infection.
4. To establish if any of the early immunological events are associated with better GCs activity, antibody quality and quantities in chronic HIV infection.

Flow cytometry was used for the determination of T and B cells phenotypes in early HIV infection. Consistent with the literature, CD4+ T cells were depleted, and this was accompanied by a depletion of CD4+ effector memory T cell compartment [220]. However, there was an expansion of memory Tfh cells despite the depletion of CD4+ T cells, similar to other reports [86, 93]. HIV related exhaustion of the CD4+ T cell compartment was also evident, where there was an upregulation of PD-1. The upregulation of PD-1 on CD4+ T cells in HIV infected individuals has been described before [224] and has been reported to limit quality antibody generation in HIV infection [86].

Additionally, different T follicular helper (Tfh) cell subsets such as ICOS+ PD-1+ CXCR5+ CD4+ and PD-1+ CXCR3- CXCR5+ CD4+ T cells had significant associations with HIV-specific antibody levels, pointing out to a coordinated GCs activity. Using HIV Envelope (ENV) peptides, I detected HIV-specific Tfh cells, and these cells were significantly associated with antibody-dependent natural killer cell - interferon-gamma *secretion* ($ADNK_{IFN-\gamma}$), which is an Fc-mediated function. Although associations to other functions did not reach significance level, Tfh frequencies increased with higher HIV-specific Fc- and Fab-mediated functions. The limited sample size may likely have influenced the power of the associations. However, these data support the inclusion of antigen-specific subsets in future larger cohort studies.

Consistent with the literature, an expansion of atypical and activated B cell subsets and a depletion of resting B cells was observed in HIV infected individuals [101]. HIV-specific memory B cells were detected, and this subset had direct correlations with class-switched and mature B cells, the cells they differentiate from. This also supports that GCs activities favour the generation of antigen-specific B cells.

When T and B interactions were explored, activated B cells directly associated with CD4+ T cells, pointing to potentially available help that B cells require in the GCs. Consistent with the literature [105, 210], memory Tfh cells positively correlated with class-switched B cells further confirming the potential role of Tfh cells, which are critical for B cell class-switching in the GCs. However, no significant association was observed between B and T cell subsets with the markers of HIV disease progression.

To determine HIV-specific antibody levels (isotypes and subclasses), an in-house enzyme-linked immunosorbent assay (ELISA) and multiplex assay were used. The multiplex assay was included to test a broader range of antigens. Also, the plasma levels of B cell activating factor (BAFF) and CXCL13 cytokines were determined before and during early HIV infection using commercial cytokine-ELISA kits. The reason for determining BAFF and CXCL13 levels before HIV infection was to establish if some individuals were intrinsically inclined to better GC reactions and hence better HIV disease outcomes.

There was a significant increase in HIV-specific antibody isotypes and subclasses antibody levels as HIV disease progressed, which may be linked with the rise of HIV viremia [287]. Consistent with the literature, immunoglobulin (Ig) A isotype circulated at low levels in plasma [288], while IgG_1 was the most predominant subclass and IgG_4 was the least dominant [256, 258, 292, 293]. HIV-specific IgM was detected up to 48 months PI, supporting a role of constant HIV antigenic stimulation at the GCs that lead to a continuous IgM generation to new variants, despite the ongoing class switching from the previously generated responses [28, 287]. Theoretically, any new mutant viruses during chronic HIV infection may be processed as a “new infection” prompting the generation of IgM antibodies.

There were positive associations between antibody levels with markers of HIV disease progression, which implies that, while the high viral load is needed to drive the GCs activity, the preservation of CD4+ T cell counts is also essential to provide B cell help. Such a balance of immunological reactions has been suggested to be a driver of the development of broadly neutralising antibodies (bnAbs) [294].

Unlike the increase in HIV-specific antibody isotypes and subclasses levels as HIV disease progressed, there was a decrease in the levels of BAFF and CXCL13 cytokines after infection, which remained low until 48 months post-infection. A previous South African cohort reported a rise in BAFF and CXCL13 levels after infection [217], contrarily to the current observation. It is possible that BAFF and CXCL13 peaked earlier than our sampling time point at 3 months PI. It is not clear why there was a decrease in these cytokines' levels, considering that HIV is associated with a cytokine storm after infection. Nevertheless, CXCL13 and BAFF levels positively correlated, suggesting, a coordinated GCs activity.

Furthermore, CXCL13 levels were directly associated with HIV viral load measurements, enhancing the role of viremia in driving GCs activities as described elsewhere [277, 278, 364]. However, BAFF had weak associations with the markers of HIV disease progression, and this has been described elsewhere [101]. Interestingly, a positive association of B cell cytokines and HIV-specific antibody levels were observed, suggesting that higher levels of B cell cytokines would drive higher levels of antigen-specific antibodies. For instance, CXCL13 levels were positively associated with HIV-specific isotypes and subclasses antibody levels. The cytokine CXCL13 organizes the B cell follicles of secondary lymphoid organs via recruiting antigen-specific B and

T cells to the GC through the CXCR5 receptor [295]; thus, its association with antibody levels is expected.

Similarly, BAFF was associated with higher levels of HIV-specific IgG. Since BAFF is essential for B cell survival and maturation [97], it is plausible that higher levels of BAFF would favour higher frequencies of B cells and hence higher levels of antigen-specific antibodies. However, data suggested that, unlike BAFF, CXCL13 was a better marker of HIV-specific antibody levels.

Flow cytometry was used to determine antibody-dependent cellular phagocytosis (ADCP), antibody-dependent complement deposition (ADCD), antibody-dependent neutrophil phagocytosis (ADNP) and natural killer cells degranulation expressed by CD107α , and secretion of cytokines as per MIP-1α and IFN-γ levels in plasma taken from HIV infected individuals. A luciferase reporter assay was used to determine the neutralising breadth and potency in plasma samples.

Similar to other adult studies, Fc-mediated antibody function in HIV infection was detectable early in infection (3 months PI) [134, 334]. It is possible that these early responses support CD8+ T cell responses in bringing down the HIV viremia to the viral set point. Fc mediated functions increased over time and this could be as a result of increasing titres of HIV-specific isotypes and subclasses antibody levels [134, 143]. Notably, individuals who made quality Fc-mediated responses in early HIV infection tended to maintain these responses as the disease progressed suggesting that early priming of Fc responses may be critical in maintaining them in the course of HIV. This may also indicate that the generation of functional Fc-mediated functions may be an intrinsic immunological process, with some individuals making them and sustaining them while others do not.

There were direct associations between the Fc effector functions measured, suggesting a coordinated Fc-mediated immune response in HIV. Such a response, though not extensively studied, has been suggested to play a role in HIV elite controllers' early control of viremia. HIV elite controllers were reported to displayed a functionally coordinated innate immune-recruiting response involving natural killer cell functions, monocyte and neutrophil phagocytosis and complement [146]. Also, Fc polyfunctionality in early HIV infection is a signature of bnAbs development in chronic HIV infection [134]. From this study, it is not clear what drives Fc polyfunctionality in HIV, since Fc polyfunctionality correlated negatively with both HIV viral load and CD4+ T cell counts. However, and consistent with the literature [342], data here suggests that ADCP may play a role in viremia control.

Unlike Fc-mediated functions, Fab-mediated function was rare. This result is similar to other reports where only 10-20% of HIV infected individuals have been shown to develop bnAbs [159, 313]. There was no association between antibody neutralisation function with viral load or CD4+ T cell counts, unlike in other studies [313, 344, 345]. The lack of association could be due to the limited sample size and that only a very small fraction of individuals developed any level of neutralisation and this limits the level of interpretation. However, when only those individuals who developed any level of breadth were considered, positive associations between ADCP scores at 3- and 12-months PI and breadth development at 48 months PI were observed. This may suggest that quality ADCP function in early HIV infection may be a driver or a signature of the development of neutralisation breadth in chronic infection. In contrast, HIV viral load measurements at 6 months PI negatively correlated with the neutralisation breadth at 48 months PI, suggesting that the high viremia in early HIV infection may lead to the failure of the selection of B cells that may further breadth development.

Indeed, only ADCD function in early HIV infection negatively associated with the development of breadth. This finding may imply that quality ADCD activity in early HIV infection may reduce viremia, hence eliminating one of the crucial drivers of bnAbs development [159]. Although the lack of strong associations between bnAbs and Fc-mediated responses, there were trends of positive associations between the two antibody functions. This may suggest that both Fc and Fab functions are intrinsically linked and modulated distinctly in the GCs, similar to previous suggestions [134]. However, when only the participants who developed any level of neutralisation were considered and associated with Fc-mediated functions, ADCP function in early HIV infection predicted the development of breadth. This may infer that quality ADCP function in early HIV infection could be a driver or a signature of bnAbs generation in chronic HIV. We observed an association between the Fc and Fab-mediated functions, although not very strong, which suggested coordinated GCs activities. An increase in the sample size may probably have provided a better power for the association.

Using a system serology approach, I associated immunological events during chronic infection with those measured earlier in the infection to establish how well these are predictive of downstream antibody functions. The Spearman's correlation values for each association and their p-values were determined and visualized using the R package *corrplot*. The variables were then regrouped according to their level of association using the "*hclust"* algorithm on R.

Memory Tfh cells in early HIV infection negatively associated with CXCL13 levels, emphasizing the role of HIV viremia in depleting CD4+ T cells and driving GCs activity [294]. Additionally, memory Tfh cells in early infection were associated with HIV-specific antibody levels in chronic

disease inferring that they are vital in offering B cells help [99, 225], which may lead to high titres of functional antibodies. Other subsets of memory Tfh cells such as PD-1+ CXCR3- CXCR5+ CD4+ T cells and ICOS+ PD-1+ CXCR5+ CD4+ T cells were associated with quality Fc effector functions and higher titres of HIV-specific antibodies. Mainly, PD-1+ CXCR3- CXCR5+ CD4+ T cells at 3 months PI had direct associations with IgG and IgG_1 levels in chronic infection. Similarly, ICOS+ PD-1+ CXCR5+ CD4+ T cells in early infection had associations with IgM and IgG levels. This suggests that these subsets drive antibody generation, as reported elsewhere [99, 357].

The association between central memory CD4+ T cells with HIV-specific antibody levels may infer that the preservation of this subset is critical in the generation of higher antibody titres. Additionally, HIV-specific Tfh cells had significant associations with $ADNK_{IFN\text{-}\gamma}$ activity indicating the role of HIV-specific Tfh cells in antibody class switching, that is essential for Fc-mediated function.

Due to the disruption of the B cell compartment by HIV [354, 355], different B cell subsets had different associations with antibody responses. Activation of B cells was required for the generation of higher titres of HIV-specific antibodies while resting B cells were negatively associated with CXCL13 levels, probably due to the effect of viremia. HIV viremia leads to the activation of resting memory B cells and drives GCs activity. Similarly, CXCL13 levels at 3 months PI correlated with antibody levels in chronic HIV infection, highlighting the role of the GCs activity in antibody generation.

The uniqueness of the current study is that it identified HIV-specific subsets of B and T cells in an ART naïve longitudinal cohort of HIV infected individuals. Additionally, this study used early

HIV-specific subsets of B and T cells to describe HIV-specific antibody levels and functions later on in infection. Since lymph node samples were unavailable, the pairing of peripheral HIV-specific cellular subsets with CXCL13 and BAFF was used to understand GCs activities [96]. Other studies either addressed general T cell subsets in longitudinal cohorts [99, 225] or used cross-sectional studies to assess the association with antibody function [93, 100, 361].

The current study addressed HIV-specific cell subsets associations with HIV-specific antibody levels and functions, as these are the cells that specifically drive the HIV-specific responses. Also, using a longitudinal cohort where HIV-specific cell subsets in early infection are associated with subsequent antibody functions and levels in chronic HIV provides a biologically accurate association. This is because antibody functions and levels are often reflective of cellular events that occurred much earlier in an infection. More novel was the association of HIV-specific cellular subsets with Fc-mediated functions, which other studies have not done, despite Fc-mediated function being the only function to be associated with protection in the RV144 vaccine clinical trial [240].

In summary, I have shown that some T and B cells subsets predict higher titres of HIV-specific antibodies in chronic HIV infection. Additionally, HIV viremia drives quality GCs activities (higher CXCL13 levels) which translates into higher levels of HIV-specific antibodies. I have also demonstrated that there exists an association between early immunological events with downstream antibody functions and levels.

This study emphasizes the need for longitudinal studies that assess antigen-specific responses, as this may provide additional information. Associating of early cellular events with downstream outcomes provides a biologically accurate association since antibody functions and levels are often

reflective of cellular events that occurred much earlier in an infection. Lastly, since Fab- and Fc-mediated functions are mechanistically linked [134], associating both functions with early cellular frequencies and GCs activities may capture the whole scope of antigen-specific responses. These findings would benefit from confirmation in studies that have access to lymphoid tissues, that would be more reflective of the early cellular events as these occur in the lymphoid organs and were inaccessible in the current study. The identification of early immunological signatures that can predict downstream immunological outcomes would contribute in shepherding desired immune responses for vaccines and vaccination strategies. Figure 7.1 summarises the objectives and findings of this study.

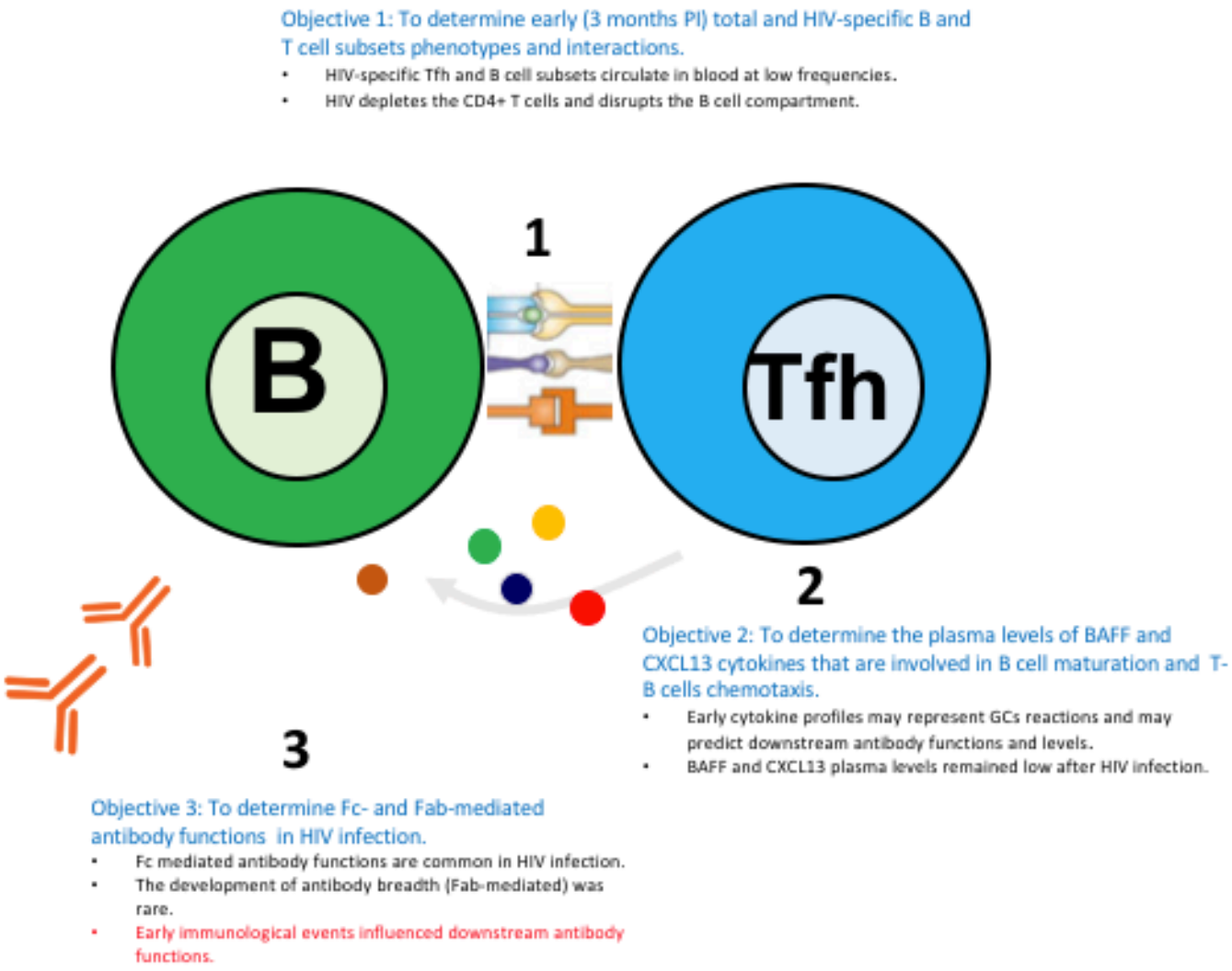


Figure 7.1: A summary of the objectives and findings of this study. In objective 1, general and HIV-specific B and T cell phenotypes were characterised at 3 months PI, and their association assessed. In objective 2, levels of B cell cytokines, CXCL13 and BAFF were determined before HIV infection and at 3-, 12- and 48 months PI. In objective 3, antibody levels and functions (Fc- and Fab-mediated) were determined at 3-, 12- and 48 months PI. Associations between the cellular phenotypes and B cell cytokines at 3 months PI were done with antibody levels and functions at 3-, 12- and 48- months PI. The numbers on the figure (1, 2 and 3) have been used to represent each objective.

This study had limitations such as:

- The lack of lymphoid tissues for the characterisation of T and B cell subsets.
- The use of a bead assay rather than the actual virus in Fc-mediated assays.
- The use of a single HIV antigen in the activation induced marker (AIM) assay.

However, the associations as described above, that is, between early phenotypic and GCs events with downstream antibody levels and functions showed that early phenotypes associated better with antibody levels, with better defined subsets improving the association. CXCL13 was also observed to be a better marker of GCs activities than BAFF and showed significant association with downstream antibody levels.

References

1. Sharp, P.M. and B.H. Hahn, *Origins of HIV and the AIDS pandemic.* Cold Spring Harbor perspectives in medicine, 2011. **1**(1): p. a006841-a006841.
2. Clavel, F., et al., *Isolation of a new human retrovirus from West African patients with AIDS.* Science, 1986. **233**(4761): p. 343-6.
3. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-71.
4. De Leys, R., et al., *Isolation and partial characterization of an unusual human immunodeficiency retrovirus from two persons of west-central African origin.* J Virol, 1990. **64**(3): p. 1207-16.
5. Simon, F., et al., *Identification of a new human immunodeficiency virus type 1 distinct from group M and group O.* Nat Med, 1998. **4**(9): p. 1032-7.
6. Plantier, J.C., et al., *A new human immunodeficiency virus derived from gorillas.* Nat Med, 2009. **15**(8): p. 871-2.
7. Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA.* Annu Rev Biochem, 1998. **67**: p. 1-25.
8. Schrager, J.A. and J.W. Marsh, *HIV-1 Nef increases T cell activation in a stimulus-dependent manner.* Proc Natl Acad Sci U S A, 1999. **96**(14): p. 8167-72.
9. Roeth, J.F., et al., *HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail.* J Cell Biol, 2004. **167**(5): p. 903-13.
10. Engelman, A. and P. Cherepanov, *The structural biology of HIV-1: mechanistic and therapeutic insights.* Nat Rev Microbiol, 2012. **10**(4): p. 279-90.

11. Arhel, N.J., et al., *HIV-1 DNA Flap formation promotes uncoating of the pre-integration complex at the nuclear pore.* Embo j, 2007. **26**(12): p. 3025-37.

12. Barreto-de-Souza, V., et al., *HIV-1 VAGINAL TRANSMISSION: CELL-FREE OR CELL-ASSOCIATED VIRUS?* American journal of reproductive immunology (New York, N.Y. : 1989), 2014. **71**(6): p. 589-599.

13. Sodora, D.L., et al., *Vaginal transmission of SIV: assessing infectivity and hormonal influences in macaques inoculated with cell-free and cell-associated viral stocks.* AIDS Res Hum Retroviruses, 1998. **14 Suppl 1**: p. S119-23.

14. Haaland, R.E., et al., *Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1.* PLoS Pathog, 2009. **5**(1): p. e1000274.

15. McMichael, A.J., et al., *The immune response during acute HIV-1 infection: clues for vaccine development.* Nat Rev Immunol, 2010. **10**(1): p. 11-23.

16. Macharia G et al., *Transmission of Multiple HIV-1 Founder Viruses and High Frequency of Unique Recombinant Forms among MSM in Kenya.* AIDS Research and Human Retroviruses. Conference: Conference on HIV Research for Prevention (HIV R4P), 2016. **32**.

17. Carlson, J.M., et al., *HIV transmission. Selection bias at the heterosexual HIV-1 transmission bottleneck.* Science, 2014. **345**(6193): p. 1254031.

18. Bar, K.J., et al., *Wide variation in the multiplicity of HIV-1 infection among injection drug users.* J Virol, 2010. **84**(12): p. 6241-7.

19. Li, Q., et al., *Glycerol monolaurate prevents mucosal SIV transmission.* Nature, 2009. **458**(7241): p. 1034-8.

20. Geijtenbeek, T.B., et al., *DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells.* Cell, 2000. **100**(5): p. 587-97.

21. Moir, S., et al., *B cells of HIV-1-infected patients bind virions through CD21-complement interactions and transmit infectious virus to activated T cells.* J Exp Med, 2000. **192**(5): p. 637-46.

22. Gasper-Smith, N., et al., *Induction of plasma (TRAIL), TNFR-2, Fas ligand, and plasma microparticles after human immunodeficiency virus type 1 (HIV-1) transmission: implications for HIV-1 vaccine design.* J Virol, 2008. **82**(15): p. 7700-10.

23. Levesque, M.C., et al., *Polyclonal B cell differentiation and loss of gastrointestinal tract germinal centers in the earliest stages of HIV-1 infection.* PLoS Med, 2009. **6**(7): p. e1000107.

24. Shelton, J.D., *Ten myths and one truth about generalised HIV epidemics.* Lancet, 2007. **370**(9602): p. 1809-11.

25. Jaffe, H.W., R.O. Valdiserri, and K.M. De Cock, *The reemerging HIV/AIDS epidemic in men who have sex with men.* Jama, 2007. **298**(20): p. 2412-4.

26. Kozlov, A.P., et al., *HIV incidence and behavioral correlates of HIV acquisition in a cohort of injection drug users in St Petersburg, Russia.* Medicine, 2016. **95**(44): p. e5238-e5238.

27. Wawer, M.J., et al., *Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda.* J Infect Dis, 2005. **191**(9): p. 1403-9.

28. Fiebig, E.W., et al., *Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection.* Aids, 2003. **17**(13): p. 1871-9.

29. Stacey, A.R., et al., *Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections.* J Virol, 2009. **83**(8): p. 3719-33.

30. Pantaleo, G., C. Graziosi, and A.S. Fauci, *The immunopathogenesis of human immunodeficiency virus infection.* N Engl J Med, 1993. **328**(5): p. 327-35.

31. Robb, M.L. and J. Ananworanich, *Lessons from acute HIV infection.* Curr Opin HIV AIDS, 2016.

32. Borrow, P., et al., *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.* J Virol, 1994. **68**(9): p. 6103-10.

33. Pantaleo, G., et al., *HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease.* Nature, 1993. **362**(6418): p. 355-8.

34. *Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California.* MMWR Morb Mortal Wkly Rep, 1981. **30**(25): p. 305-8.

35. *Update on acquired immune deficiency syndrome (AIDS)--United States.* MMWR Morb Mortal Wkly Rep, 1982. **31**(37): p. 507-8, 513-4.

36. WHO., *Interim WHO clinical staging of HVI/AIDS and HIV/AIDS case definitions for surveillance: African Region. 2005, Geneva: World Health Organization.*

. UNAIDS, Global HIV & AIDS statistics — 2020 fact sheet. (Searched on May 31st 2020). 2005.

37. UNAIDS, *Global HIV & AIDS statistics — 2020 fact sheet.* https://www.unaids.org/en/resources/fact-sheet (Acessed on June 30th 2020), 2020.

38. WHO., *Coronavirus disease (COVID-19) pandemic.* Accessed at www.who.int/emergencies/diseases/novel-coronavirus-2019 on 30 June 2020., 2020.

39. Haerter, G., et al., *COVID-19 in people living with human immunodeficiency virus: A case series of 33 patients.* medRxiv, 2020: p. 2020.04.28.20073767.

40. Blanco, J.L., et al., *COVID-19 in patients with HIV: clinical case series.* The lancet. HIV, 2020. **7**(5): p. e314-e316.

41. Guo, W.a.M., Fangzhao and Dong, Yu and Zhang, Qian and Zhang, Xiaoxia and Mo, Pingzheng and Feng, Yong and Liang, Ke., *A Survey for COVID-19 Among HIV/AIDS Patients in Two Districts of Wuhan, China (3/4/2020).* . 2020. **Available at SSRN: https://ssrn.com/abstract=3550029 or http://dx.doi.org/10.2139/ssrn.3550029**

42. Wang, W., et al., *High-dimensional immune profiling by mass cytometry revealed immunosuppression and dysfunction of immunity in COVID-19 patients.* Cellular & molecular immunology, 2020. **17**(6): p. 650-652.

43. Borobia, A.M., et al., *A Cohort of Patients with COVID-19 in a Major Teaching Hospital in Europe.* Journal of clinical medicine, 2020. **9**(6): p. 1733.

44. *Comparison of female to male and male to female transmission of HIV in 563 stable couples. European Study Group on Heterosexual Transmission of HIV.* Bmj, 1992. **304**(6830): p. 809-13.

45. Kirtley, S. and P. Chien, *Women out loud: how women living with HIV will help the world end AIDS.* BJOG: an international journal of obstetrics and gynaecology, 2013. **120**(5): p. 652.

46. de Vincenzi, I., *Triple antiretroviral compared with zidovudine and single-dose nevirapine prophylaxis during pregnancy and breastfeeding for prevention of mother-to-*

child transmission of HIV-1 (Kesho Bora study): a randomised controlled trial. Lancet Infect Dis, 2011. **11**(3): p. 171-80.

47. Chasela, C.S., et al., *Maternal or infant antiretroviral drugs to reduce HIV-1 transmission.* N Engl J Med, 2010. **362**(24): p. 2271-81.

48. Jamieson, D.J., et al., *Maternal and infant antiretroviral regimens to prevent postnatal HIV-1 transmission: 48-week follow-up of the BAN randomised controlled trial.* Lancet, 2012. **379**(9835): p. 2449-2458.

49. Fonner, V.A., et al., *Effectiveness and safety of oral HIV preexposure prophylaxis for all populations.* Aids, 2016. **30**(12): p. 1973-83.

50. Pinkerton, S.D., D.R. Holtgrave, and H.J. Pinkerton, *Cost-effectiveness of chemoprophylaxis after occupational exposure to HIV.* Arch Intern Med, 1997. **157**(17): p. 1972-80.

51. Chersich, M.F. and H.V. Rees, *Vulnerability of women in southern Africa to infection with HIV: biological determinants and priority health sector interventions.* Aids, 2008. **22 Suppl 4**: p. S27-40.

52. Zaidi, J., et al., *Dramatic increase in HIV prevalence after scale-up of antiretroviral treatment.* AIDS (London, England), 2013. **27**(14): p. 2301-2305.

53. UNAIDS, *Global HIV and AIDS statistics - 2019 fact sheet.* https://www.unaids.org/en/resources/fact-sheet, 2019. **Global Report 2019 - Accessed March 1, 2020.**

54. WHO., *Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach, 2nd ed. World*

Health Organization. https://apps.who.int/iris/handle/10665/208825 (Searched on 14th April 2020), 2016.

55. Organization, W.H., *The use of antiretroviral drugs for treating and preventing HIV infection.* 2016. **Second edition**.

56. Kemnic TR, G.P., *HIV Antiretroviral Therapy. [Updated 2020 Jun 23]. In: StatPearls [Internet].* . Treasure Island (FL): StatPearls Publishing; 2020 Jan, 2020.

57. Wilkinson, G.R., *Cytochrome P4503A (CYP3A) metabolism: prediction of in vivo activity in humans.* J Pharmacokinet Biopharm, 1996. **24**(5): p. 475-90.

58. Lapenta, C., et al., *Type I interferon is a powerful inhibitor of in vivo HIV-1 infection and preserves human CD4(+) T cells from virus-induced depletion in SCID mice transplanted with human cells.* Virology, 1999. **263**(1): p. 78-88.

59. Manches, O., et al., *HIV-activated human plasmacytoid DCs induce Tregs through an indoleamine 2,3-dioxygenase-dependent mechanism.* J Clin Invest, 2008. **118**(10): p. 3431-9.

60. Reeves, R.K., et al., *CD16- natural killer cells: enrichment in mucosal and secondary lymphoid tissues and altered function during chronic SIV infection.* Blood, 2010. **115**(22): p. 4439-46.

61. Wang, Y.E., et al., *Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1.* J Virol, 2009. **83**(4): p. 1845-55.

62. Goonetilleke, N., et al., *The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection.* J Exp Med, 2009. **206**(6): p. 1253-72.

63. Jia, M., et al., *Preferential CTL targeting of Gag is associated with relative viral control in long-term surviving HIV-1 infected former plasma donors from China.* Cell Research, 2012. **22**(5): p. 903-914.

64. Jin, X., et al., *Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques.* J Exp Med, 1999. **189**(6): p. 991-8.

65. Trimble LA and L. J., *Circulating CD8 T lymphocytes in human immunodeficiency virusinfected individuals have impaired function and downmodulate CD3 zeta, the signaling chain of the T-cell receptor complex.* Blood, 1998. **1998;91:585- 594**.

66. Salazar-Gonzalez, J.F., et al., *Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection.* J Exp Med, 2009. **206**(6): p. 1273-89.

67. Petrovas, C., et al., *Follicular CD8 T cells accumulate in HIV infection and can kill infected cells in vitro via bispecific antibodies.* Science translational medicine, 2017. **9**(373): p. eaag2285.

68. Devergne, O., et al., *Activation of cytotoxic cells in hyperplastic lymph nodes from HIV-infected patients.* Aids, 1991. **5**(9): p. 1071-9.

69. Tenner-Racz, K., et al., *Cytotoxic effector cell granules recognized by the monoclonal antibody TIA-1 are present in CD8+ lymphocytes in lymph nodes of human immunodeficiency virus-1-infected patients.* Am J Pathol, 1993. **142**(6): p. 1750-8.

70. Gratton, S., et al., *Highly restricted spread of HIV-1 and multiply infected cells within splenic germinal centers.* Proc Natl Acad Sci U S A, 2000. **97**(26): p. 14566-71.

71. Connick, E., et al., *CTL fail to accumulate at sites of HIV-1 replication in lymphoid tissue.* J Immunol, 2007. **178**(11): p. 6975-83.

72. He, R., et al., *Follicular CXCR5- expressing CD8(+) T cells curtail chronic viral infection.* Nature, 2016. **537**(7620): p. 412-428.

73. Leong, Y.A., et al., *CXCR5(+) follicular cytotoxic T cells control viral infection in B cell follicles.* Nat Immunol, 2016. **17**(10): p. 1187-96.

74. Oxenius, A., et al., *Early highly active antiretroviral therapy for acute HIV-1 infection preserves immune function of CD8+ and CD4+ T lymphocytes.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3382-7.

75. Crotty, S., *Follicular helper CD4 T cells (TFH).* Annu Rev Immunol, 2011. **29**: p. 621-63.

76. Crotty, S., *T follicular helper cell differentiation, function, and roles in disease.* Immunity, 2014. **41**(4): p. 529-42.

77. Rothchild, A.C., et al., *Role of Granulocyte-Macrophage Colony-Stimulating Factor Production by T Cells during Mycobacterium tuberculosis Infection.* MBio, 2017. **8**(5).

78. Omiya, R., et al., *Inhibition of EBV-induced lymphoproliferation by CD4(+) T cells specific for an MHC class II promiscuous epitope.* J Immunol, 2002. **169**(4): p. 2172-9.

79. Aslan, N., et al., *Cytotoxic CD4 T cells in viral hepatitis.* J Viral Hepat, 2006. **13**(8): p. 505-14.

80. Nemes, E., et al., *Cytotoxic granule release dominates gag-specific CD4+ T-cell response in different phases of HIV infection.* Aids, 2010. **24**(7): p. 947-57.

81. Yu, D., et al., *The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment.* Immunity, 2009. **31**(3): p. 457-68.

82. Nurieva, R.I., et al., *Bcl6 mediates the development of T follicular helper cells.* Science, 2009. **325**(5943): p. 1001-5.

83. Schaerli, P., et al., *CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function.* J Exp Med, 2000. **192**(11): p. 1553-62.

84. Stamatatos, L., et al., *Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine?* Nat Med, 2009. **15**(8): p. 866-70.

85. Burton, D.R., et al., *Broadly neutralizing antibodies present new prospects to counter highly antigenically diverse viruses.* Science, 2012. **337**(6091): p. 183-6.

86. Cubas, R.A., et al., *Inadequate T follicular cell help impairs B cell immunity during HIV infection.* Nat Med, 2013. **19**(4): p. 494-9.

87. Moukambi, F., et al., *CD4 T Follicular Helper Cells and HIV Infection: Friends or Enemies?* Frontiers in Immunology, 2017. **8**: p. 135.

88. Moukambi, F., et al., *Correction: Early Loss of Splenic Tfh Cells in SIV-Infected Rhesus Macaques.* PLoS Pathogens, 2016. **12**(1): p. e1005393.

89. Good-Jacobson, K.L., et al., *PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells.* Nat Immunol, 2010. **11**(6): p. 535-42.

90. Folkvord, J.M., C. Armon, and E. Connick, *Lymphoid follicles are sites of heightened human immunodeficiency virus type 1 (HIV-1) replication and reduced antiretroviral effector mechanisms.* AIDS Res Hum Retroviruses, 2005. **21**(5): p. 363-70.

91. Choi, Y.S., et al., *Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory.* J Immunol, 2013. **190**(8): p. 4014-26.

92. Yue, F.Y., et al., *HIV-specific IL-21 producing CD4+ T cells are induced in acute and chronic progressive HIV infection and are associated with relative viral control.* J Immunol, 2010. **185**(1): p. 498-506.

93. Lindqvist, M., et al., *Expansion of HIV-specific T follicular helper cells in chronic HIV infection.* J Clin Invest, 2012. **122**(9): p. 3271-80.

94. Morita, R., et al., *Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion.* Immunity, 2011. **34**(1): p. 108-21.

95. Havenith, S.H., et al., *CXCR5+CD4+ follicular helper T cells accumulate in resting human lymph nodes and have superior B cell helper activity.* Int Immunol, 2014. **26**(3): p. 183-92.

96. Havenar-Daughton, C., et al., *CXCL13 is a plasma biomarker of germinal center activity.* Proceedings of the National Academy of Sciences, 2016. **113**(10): p. 2702-2707.

97. Mackay, F., et al., *BAFF AND APRIL: a tutorial on B cell survival.* Annu Rev Immunol, 2003. **21**: p. 231-64.

98. Locci, M., et al., *Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses.* Immunity, 2013. **39**(4): p. 758-69.

99. Cohen, K., et al., *Early Preservation of CXCR5(+) PD-1(+) Helper T Cells and B Cell Activation Predict the Breadth of Neutralizing Antibody Responses in Chronic HIV-1 Infection.* Journal of Virology, 2014. **88**(22): p. 13310-13321.

100. Ranasinghe, S., et al., *HIV-1 Antibody Neutralization Breadth Is Associated with Enhanced HIV-Specific CD4+ T Cell Responses.* J Virol, 2015. **90**(5): p. 2208-20.

101. Mabuka, J.M., et al., *Plasma CXCL13 but Not B Cell Frequencies in Acute HIV Infection Predicts Emergence of Cross-Neutralizing Antibodies.* Frontiers in Immunology, 2017. **8**: p. 1104.

102. Saifi M and W. CA., *Autoimmune Disease in Primary Immunodeficiency: At the Crossroads of Anti- Infective Immunity and Self-Tolerance.* Immunol Allergy Clin North Am, 2015. **35:731–752.**

103. Kardava, L., et al., *Abnormal B cell memory subsets dominate HIV-specific responses in infected individuals.* J Clin Invest, 2014. **124**(7): p. 3252-62.

104. Wheatley, A.K., et al., *HIV-dependent depletion of influenza-specific memory B cells impacts B cell responsiveness to seasonal influenza immunisation.* Sci Rep, 2016. **6**: p. 26478.

105. De Silva, N.S. and U. Klein, *Dynamics of B cells in germinal centres.* Nat Rev Immunol, 2015. **15**(3): p. 137-48.

106. Forthal, D.N., G. Landucci, and E.S. Daar, *Antibody from patients with acute human immunodeficiency virus (HIV) infection inhibits primary strains of HIV type 1 in the presence of natural-killer effector cells.* J Virol, 2001. **75**(15): p. 6953-61.

107. Miller, C.J., et al., *Antiviral antibodies are necessary for control of simian immunodeficiency virus replication.* J Virol, 2007. **81**(10): p. 5024-35.

108. Gaufin, T., et al., *Limited ability of humoral immune responses in control of viremia during infection with SIVsmmD215 strain.* Blood, 2009. **113**(18): p. 4250-61.

109. Buckner, C.M., et al., *Maintenance of HIV-Specific Memory B-Cell Responses in Elite Controllers Despite Low Viral Burdens.* J Infect Dis, 2016. **214**(3): p. 390-8.

110. Landais, E., et al., *Broadly Neutralizing Antibody Responses in a Large Longitudinal Sub-Saharan HIV Primary Infection Cohort.* PLoS pathogens, 2016. **12**(1): p. e1005369-e1005369.

111. Gray, E.S., et al., *Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection.* J Virol, 2007. **81**(12): p. 6187-96.

112. Binley, J.M., et al., *Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients chronically infected with human immunodeficiency virus type 1 subtypes B and C.* J Virol, 2008. **82**(23): p. 11651-68.

113. Richman, D.D., et al., *Rapid evolution of the neutralizing antibody response to HIV type 1 infection.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4144-9.

114. Wei, X., et al., *Antibody neutralization and escape by HIV-1.* Nature, 2003. **422**(6929): p. 307-12.

115. Scamurra, R.W., et al., *Mucosal plasma cell repertoire during HIV-1 infection.* J Immunol, 2002. **169**(7): p. 4008-16.

116. Popovic, M., et al., *Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS.* Science, 1984. **224**(4648): p. 497-500.

117. Simek, M.D., et al., *Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm.* J Virol, 2009. **83**(14): p. 7337-48.

118. Yates, N.L., et al., *Multiple HIV-1-specific IgG3 responses decline during acute HIV-1: implications for detection of incident HIV infection.* Aids, 2011. **25**(17): p. 2089-97.

119. Moir, S. and A.S. Fauci, *B cells in HIV infection and disease.* Nat Rev Immunol, 2009. **9**(4): p. 235-45.

120. Lane, H.C., et al., *Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome.* N Engl J Med, 1983. **309**(8): p. 453-8.

121. Martínez-Maza, O. and E.C. Breen, *B-cell activation and lymphoma in patients with HIV.* Curr Opin Oncol, 2002. **14**(5): p. 528-32.

122. Moir, S., et al., *Decreased survival of B cells of HIV-viremic patients mediated by altered expression of receptors of the TNF superfamily.* J Exp Med, 2004. **200**(5): p. 587-99.

123. Haynes, B.F., et al., *Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies.* Science, 2005. **308**(5730): p. 1906-8.

124. Ferretti, E., et al., *Interleukin-17A promotes the growth of human germinal center derived non-Hodgkin B cell lymphoma.* Oncoimmunology, 2015. **4**(10): p. e1030560.

125. Gray, E.S., et al., *Broad neutralization of human immunodeficiency virus type 1 mediated by plasma antibodies against the gp41 membrane proximal external region.* J Virol, 2009. **83**(21): p. 11265-74.

126. Parren, P.W., et al., *Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro.* J Virol, 2001. **75**(17): p. 8340-7.

127. Burton, D.R., et al., *Limited or no protection by weakly or nonneutralizing antibodies against vaginal SHIV challenge of macaques compared with a strongly neutralizing antibody.* Proc Natl Acad Sci U S A, 2011. **108**(27): p. 11181-6.

128. Baba, T.W., et al., *Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection.* Nat Med, 2000. **6**(2): p. 200-6.

129. Simonich, C.A., et al., *HIV-1 neutralizing antibodies with limited hypermutation from an infant.* Cell, 2016. **166**(1): p. 77-87.

130. Liu, M., et al., *Polyreactivity and autoreactivity among HIV-1 antibodies.* J Virol, 2015. **89**(1): p. 784-98.

131. Klein, F., et al., *HIV therapy by a combination of broadly neutralizing antibodies in humanized mice.* Nature, 2012. **492**(7427): p. 118-22.

132. Trkola, A., et al., *Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies.* Nat Med, 2005. **11**(6): p. 615-22.

133. Boswell, K.L., et al., *Loss of circulating CD4 T cells with B cell helper function during chronic HIV infection.* PLoS Pathog, 2014. **10**(1): p. e1003853.

134. Richardson, S.I., et al., *HIV-specific Fc effector function early in infection predicts the development of broadly neutralizing antibodies.* PLoS pathogens, 2018. **14**(4): p. e1006987-e1006987.

135. Robinson, H.L., *Non-neutralizing antibodies in prevention of HIV infection.* Expert Opinion on Biological Therapy, 2013. **13**(2): p. 197-207.

136. Chung, A., et al., *The utility of ADCC responses in HIV infection.* Curr HIV Res, 2008. **6**(6): p. 515-9.

137. Tyler DS, Nastala CL, and S. SD, *GP120 specific cellular cytotoxicity in HIV-1 seropositive individuals. Evidence for circu- lating CD16+ effector cells armed in vivo with cytophilic antibody.* Journal of Immunology, 1989. **142**: p. 1177-82.

138. Mavilio, D., et al., *Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 15011-6.

139. Giese, S. and M. Marsh, *Tetherin can restrict cell-free and cell-cell transmission of HIV from primary macrophages to T cells.* PLoS Pathog, 2014. **10**(7): p. e1004189.

140. Sauter, D., A. Specht, and F. Kirchhoff, *Tetherin: holding on and letting go.* Cell, 2010. **141**(3): p. 392-8.

141. Parato, K.G., et al., *Normalization of natural killer cell function and phenotype with effective anti-HIV therapy and the role of IL-10.* Aids, 2002. **16**(9): p. 1251-6.

142. Haynes , B.F., et al., *Immune-Correlates Analysis of an HIV-1 Vaccine Efficacy Trial.* New England Journal of Medicine, 2012. **366**(14): p. 1275-1286.

143. Baum, L.L., et al., *HIV-1 gp120-specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression.* J Immunol, 1996. **157**(5): p. 2168-73.

144. Lambotte, O., et al., *Heterogeneous neutralizing antibody and antibody-dependent cell cytotoxicity responses in HIV-1 elite controllers.* Aids, 2009. **23**(8): p. 897-906.

145. Mabuka, J., et al., *HIV-specific antibodies capable of ADCC are common in breastmilk and are associated with reduced risk of transmission in women with high viral loads.* PLoS Pathog, 2012. **8**(6): p. e1002739.

146. Ackerman, M.E., et al., *Polyfunctional HIV-Specific Antibody Responses Are Associated with Spontaneous HIV Control.* PLoS Pathog, 2016. **12**(1): p. e1005315.

147. Hessell, A.J., et al., *Fc receptor but not complement binding is important in antibody protection against HIV.* Nature, 2007. **449**(7158): p. 101-4.

148. Taylor, B.S., et al., *The challenge of HIV-1 subtype diversity.* N Engl J Med, 2008. **358**(15): p. 1590-602.

149. Robinson, H.L., *HIV/AIDS Vaccines: 2018.* Clinical pharmacology and therapeutics, 2018. **104**(6): p. 1062-1073.

150. Gilbert, P.B., et al., *Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial.* J Infect Dis, 2005. **191**(5): p. 666-77.

151. Pitisuttithum, P., et al., *Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand.* J Infect Dis, 2006. **194**(12): p. 1661-71.

152. Buchbinder, S.P., et al., *Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial.* Lancet, 2008. **372**(9653): p. 1881-1893.

153. Gray, G.E., et al., *Safety and efficacy of the HVTN 503/Phambili study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study.* Lancet Infect Dis, 2011. **11**(7): p. 507-15.

154. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.* N Engl J Med, 2009. **361**(23): p. 2209-20.

155. Hammer, S.M., et al., *Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine.* N Engl J Med, 2013. **369**(22): p. 2083-92.

156. Koup, R.A., et al., *Priming immunization with DNA augments immunogenicity of recombinant adenoviral vectors for both HIV-1 specific antibody and T-cell responses.* PLoS One, 2010. **5**(2): p. e9015.

157. UNAIDS, *HVTN 702 clinical trial of an HIV vaccine stopped.* https://www.unaids.org/en/resources/presscentre/pressreleaseandstatementarchive/2020/february/20200204_vaccine, 2020. **(Accessed on March 5th 2020)**.

158. WHO., *Antiretroviral therapy for HIV infection in adults and adolescents - Recommendations for a public health approach (2006 revision).* https://www.who.int/hiv/pub/arv/adult/en/ (Accessed on April 30th 2020), 2006.

159. Landais, E., et al., *Broadly Neutralizing Antibody Responses in a Large Longitudinal Sub-Saharan HIV Primary Infection Cohort.* PLoS Pathog, 2016. **12**(1): p. e1005369.

160. Cohen, J., *Statistical Power Analysis for the Behavioral Sciences, Second Edition*. 1988: Mahwah, NJ: Lawrence Erlbaum Associates.

161. Havenar-Daughton, C., et al., *Cytokine-Independent Detection of Antigen-Specific Germinal Center T Follicular Helper Cells in Immunized Nonhuman Primates Using a Live Cell Activation-Induced Marker Technique.* J Immunol, 2016. **197**(3): p. 994-1002.

162. Dan, J.M., et al., *A Cytokine-Independent Approach To Identify Antigen-Specific Human Germinal Center T Follicular Helper Cells and Rare Antigen-Specific CD4+ T Cells in Blood.* J Immunol, 2016. **197**(3): p. 983-93.

163. Ackerman, M.E., et al., *A robust, high-throughput assay to determine the phagocytic activity of clinical antibody samples.* J Immunol Methods, 2011. **366**(1-2): p. 8-19.

164. Li, M., et al., *Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies.* J Virol, 2005. **79**(16): p. 10108-25.

165. Simek, M.D., et al., *Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput*

neutralization assay together with an analytical selection algorithm. Journal of virology, 2009. **83**(14): p. 7337-7348.

166. Flint, S.J.E., W.; Racaniello, V.R.; Skalka, A.M. , *Virological Methods.* Principles of Virology. ASM Press. ISBN 978-1-55581-443-4., 2009.

167. Liu, Y., et al., *Gene-targeted B-deficient mice reveal a critical role for B cells in the CD4 T cell response.* Int Immunol, 1995. **7**(8): p. 1353-62.

168. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells.* Nat Rev Immunol, 2010. **10**(3): p. 170-81.

169. Corinti, S., et al., *Regulatory activity of autocrine IL-10 on dendritic cell functions.* J Immunol, 2001. **166**(7): p. 4312-8.

170. Melchers, F., *Checkpoints that control B cell development.* The Journal of clinical investigation, 2015. **125**(6): p. 2203-2210.

171. Brack, C., et al., *A complete immunoglobulin gene is created by somatic recombination.* Cell, 1978. **15**(1): p. 1-14.

172. Nutt, S.L., et al., *Commitment to the B-lymphoid lineage depends on the transcription factor Pax5.* Nature, 1999. **401**(6753): p. 556-62.

173. LeBien, T.W. and T.F. Tedder, *B lymphocytes: how they develop and function.* Blood, 2008. **112**(5): p. 1570-1580.

174. Kenneth Murphy PT, M.W., *Chapter 9: The Humoral Immune Response. Janeway's Immunobiology;Seventh edition:379.*

175. Malaspina, A., et al., *Deleterious effect of HIV-1 plasma viremia on B cell costimulatory function.* J Immunol, 2003. **170**(12): p. 5965-72.

176. De Milito, A., et al., *Mechanisms of hypergammaglobulinemia and impaired antigen-specific humoral immunity in HIV-1 infection.* Blood, 2004. **103**(6): p. 2180-6.

177. Martínez-Maza, O., et al., *Infection with the human immunodeficiency virus (HIV) is associated with an in vivo increase in B lymphocyte activation and immaturity.* J Immunol, 1987. **138**(11): p. 3720-4.

178. Banchereau, J., et al., *The CD40 antigen and its ligand.* Annu Rev Immunol, 1994. **12**: p. 881-922.

179. Williams, L.D., et al., *Interleukin-21-producing HIV-1-specific CD8 T cells are preferentially seen in elite controllers.* J Virol, 2011. **85**(5): p. 2316-24.

180. Chevalier, M.F., et al., *HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function.* J Virol, 2011. **85**(2): p. 733-41.

181. Illera, V.A., et al., *Apoptosis in splenic B lymphocytes. Regulation by protein kinase C and IL-4.* J Immunol, 1993. **151**(6): p. 2965-73.

182. Beurel, E. and R.S. Jope, *Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain.* J Neuroinflammation, 2009. **6**: p. 9.

183. Giroux, M., M. Schmidt, and A. Descoteaux, *IFN-gamma-induced MHC class II expression: transactivation of class II transactivator promoter IV by IFN regulatory factor-1 is regulated by protein kinase C-alpha.* J Immunol, 2003. **171**(8): p. 4187-94.

184. Thieu, V.T., et al., *IL-4-stimulated NF-kappaB activity is required for Stat6 DNA binding.* J Leukoc Biol, 2007. **82**(2): p. 370-9.

185. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.

186. Taniguchi, T. and A. Takaoka, *The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors.* Curr Opin Immunol, 2002. **14**(1): p. 111-6.

187. Bernasconi, N.L., N. Onai, and A. Lanzavecchia, *A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells.* Blood, 2003. **101**(11): p. 4500-4.

188. Pasare, C. and R. Medzhitov, *Control of B-cell responses by Toll-like receptors.* Nature, 2005. **438**(7066): p. 364-8.

189. Lau, C.M., et al., *RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement.* J Exp Med, 2005. **202**(9): p. 1171-7.

190. Leadbetter, E.A., et al., *Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors.* Nature, 2002. **416**(6881): p. 603-7.

191. Hornung, V., et al., *Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides.* J Immunol, 2002. **168**(9): p. 4531-7.

192. Bekeredjian-Ding, I. and G. Jego, *Toll-like receptors--sentries in the B-cell response.* Immunology, 2009. **128**(3): p. 311-323.

193. Li, X., S. Jiang, and R.I. Tapping, *Toll-like receptor signaling in cell proliferation and survival.* Cytokine, 2010. **49**(1): p. 1-9.

194. Luckheeram, R.V., et al., *CD4(+)T cells: differentiation and functions.* Clin Dev Immunol, 2012. **2012**: p. 925135.

195. Janeway CA Jr, T.P., Walport M, et al., *Immunobiology: The Immune System in Health and Disease. 5th edition.* New York: Garland Science, 2001.

196. Takahama, Y., *Journey through the thymus: stromal guides for T-cell development and selection.* Nat Rev Immunol, 2006. **6**(2): p. 127-35.

197. Klein, L., et al., *Antigen presentation in the thymus for positive selection and central tolerance induction.* Nat Rev Immunol, 2009. **9**(12): p. 833-44.

198. Daniels, M.A., et al., *Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling.* Nature, 2006. **444**(7120): p. 724-9.

199. Drayton, D.L., et al., *Lymphoid organ development: from ontogeny to neogenesis.* Nat Immunol, 2006. **7**(4): p. 344-53.

200. Tao, X., et al., *Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation.* J Immunol, 1997. **159**(12): p. 5956-63.

201. Ashkar, S., et al., *Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity.* Science, 2000. **287**(5454): p. 860-4.

202. Vogelzang, A., et al., *A fundamental role for interleukin-21 in the generation of T follicular helper cells.* Immunity, 2008. **29**(1): p. 127-37.

203. Nurieva, R.I., et al., *Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages.* Immunity, 2008. **29**(1): p. 138-49.

204. Ma, C.S., et al., *Functional STAT3 deficiency compromises the generation of human T follicular helper cells.* Blood, 2012. **119**(17): p. 3997-4008.

205. Bauquet, A.T., et al., *The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells.* Nat Immunol, 2009. **10**(2): p. 167-75.

206. Johnston, R.J., et al., *STAT5 is a potent negative regulator of TFH cell differentiation.* J Exp Med, 2012. **209**(2): p. 243-50.

207. Huber, M., et al., *IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype.* Proc Natl Acad Sci U S A, 2008. **105**(52): p. 20846-51.

208. Chen, Q., et al., *IRF-4-binding protein inhibits interleukin-17 and interleukin-21 production by controlling the activity of IRF-4 transcription factor.* Immunity, 2008. **29**(6): p. 899-911.

209. Akiba, H., et al., *The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo.* J Immunol, 2005. **175**(4): p. 2340-8.

210. Victora, G.D., et al., *Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter.* Cell, 2010. **143**(4): p. 592-605.

211. Heesters, B.A., R.C. Myers, and M.C. Carroll, *Follicular dendritic cells: dynamic antigen libraries.* Nat Rev Immunol, 2014. **14**(7): p. 495-504.

212. Allen, C.D., T. Okada, and J.G. Cyster, *Germinal-center organization and cellular dynamics.* Immunity, 2007. **27**(2): p. 190-202.

213. Ansel, K.M., et al., *A chemokine-driven positive feedback loop organizes lymphoid follicles.* Nature, 2000. **406**(6793): p. 309-14.

214. Weinstein, J.S., et al., *TFH cells progressively differentiate to regulate the germinal center response.* Nat Immunol, 2016. **17**(10): p. 1197-1205.

215. Shulman, Z., et al., *Dynamic signaling by T follicular helper cells during germinal center B cell selection.* Science, 2014. **345**(6200): p. 1058-62.

216. Yan, L., et al., *T Follicular Helper Cells As a New Target for Immunosuppressive Therapies.* Frontiers in immunology, 2017. **8**: p. 1510-1510.

217. Mabuka, J.M., et al., *Plasma CXCL13 but Not B Cell Frequencies in Acute HIV Infection Predicts Emergence of Cross-Neutralizing Antibodies.* Frontiers in immunology, 2017. **8**: p. 1104-1104.

218. Moir, S. and A.S. Fauci, *B-cell responses to HIV infection.* Immunological reviews, 2017. **275**(1): p. 33-48.

219. Muema, D.M., et al., *Proportions of circulating follicular helper T cells are reduced and correlate with memory B cells in HIV-infected children.* PLoS One, 2017. **12**(4): p. e0175570.

220. Kerdiles, Y.M., et al., *Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor.* Nat Immunol, 2009. **10**(2): p. 176-84.

221. Locci, M., et al., *Human Circulating PD-1(+)CXCR3(−)CXCR5(+) Memory Tfh Cells Are Highly Functional and Correlate with Broadly Neutralizing HIV Antibody Responses.* Immunity, 2013. **39**(4): p. 758-769.

222. Rosenberg, E.S., et al., *Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia.* Science, 1997. **278**(5342): p. 1447-50.

223. Veazey, R.S., et al., *Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion.* Nature, 2005. **438**(7064): p. 99-102.

224. Foldi, J., et al., *HIV-Infected Children Have Elevated Levels of PD-1+ Memory CD4 T Cells With Low Proliferative Capacity and High Inflammatory Cytokine Effector Functions.* J Infect Dis, 2017. **216**(6): p. 641-650.

225. Locci, M., et al., *Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses.* Immunity, 2013. **39**(4): p. 758-769.

226. Vingerhoets, J., et al., *Superantigen activation of CD4+ and CD8+T cells from HIV-infected subjects: role of costimulatory molecules and antigen-presenting cells (APC).* Clin Exp Immunol, 1998. **111**(1): p. 12-9.

227. Deeks, S.G., E. Verdin, and J.M. McCune, *Immunosenescence and HIV.* Current Opinion in Immunology, 2012. **24**(4): p. 501-506.

228. Kacani, L., et al., *Detachment of human immunodeficiency virus type 1 from germinal centers by blocking complement receptor type 2.* J Virol, 2000. **74**(17): p. 7997-8002.

229. Titanji, K., et al., *Loss of memory B cells impairs maintenance of long-term serologic memory during HIV-1 infection.* Blood, 2006. **108**(5): p. 1580-7.

230. Irungu, E., et al., *Immune response to hepatitis B virus vaccination among HIV-1 infected and uninfected adults in Kenya.* The Journal of infectious diseases, 2013. **207**(3): p. 402-410.

231. Kim, H.N., et al., *Hepatitis B vaccination in HIV-infected adults: current evidence, recommendations and practical considerations.* Int J STD AIDS, 2009. **20**(9): p. 595-600.

232. Sutcliffe, C.G. and W.J. Moss, *Do children infected with HIV receiving HAART need to be revaccinated?* Lancet Infect Dis, 2010. **10**(9): p. 630-42.

233. Nduati, E.W., et al., *HIV-Exposed Uninfected Infants Show Robust Memory B-Cell Responses in Spite of a Delayed Accumulation of Memory B Cells: an Observational*

Study in the First 2 Years of Life. Clinical and vaccine immunology : CVI, 2016. **23**(7): p. 576-585.

234. Brenna, E., et al., *CD4(+) T Follicular Helper Cells in Human Tonsils and Blood Are Clonally Convergent but Divergent from Non-Tfh CD4(+) Cells.* Cell Rep, 2020. **30**(1): p. 137-152.e5.

235. Pastor, L., et al., *Dynamics of CD4 and CD8 T-Cell Subsets and Inflammatory Biomarkers during Early and Chronic HIV Infection in Mozambican Adults.* Frontiers in Immunology, 2018. **8**(1925).

236. Mascola, J.R., et al., *Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies.* Nat Med, 2000. **6**(2): p. 207-10.

237. Hessell, A.J., et al., *Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques.* Nat Med, 2009. **15**(8): p. 951-4.

238. Moldt, B., et al., *Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo.* Proc Natl Acad Sci U S A, 2012. **109**(46): p. 18921-5.

239. Shingai, M., et al., *Passive transfer of modest titers of potent and broadly neutralizing anti-HIV monoclonal antibodies block SHIV infection in macaques.* J Exp Med, 2014. **211**(10): p. 2061-74.

240. Haynes, B.F., et al., *Immune-correlates analysis of an HIV-1 vaccine efficacy trial.* N Engl J Med, 2012. **366**(14): p. 1275-86.

241. Parsons, M.S., A.W. Chung, and S.J. Kent, *Importance of Fc-mediated functions of anti-HIV-1 broadly neutralizing antibodies.* Retrovirology, 2018. **15**(1): p. 58.

242. Mayr, L.M., B. Su, and C. Moog, *Non-Neutralizing Antibodies Directed against HIV and Their Functions.* Front Immunol, 2017. **8**: p. 1590.

243. Lewis, G.K., et al., *Beyond Viral Neutralization.* AIDS Res Hum Retroviruses, 2017. **33**(8): p. 760-764.

244. Su, B. and C. Moog, *Which Antibody Functions are Important for an HIV Vaccine?* Front Immunol, 2014. **5**: p. 289.

245. Lofano, G., et al., *Antigen-specific antibody Fc glycosylation enhances humoral immunity via the recruitment of complement.* Science immunology, 2018. **3**(26): p. eaat7796.

246. Victora, G.D. and M.C. Nussenzweig, *Germinal centers.* Annu Rev Immunol, 2012. **30**: p. 429-57.

247. Ansel, K.M., et al., *A chemokine-driven positive feedback loop organizes lymphoid follicles.* Nature, 2000. **406**(6793): p. 309-14.

248. Schneider, P. and J. Tschopp, *BAFF and the regulation of B cell survival.* Immunol Lett, 2003. **88**(1): p. 57-62.

249. Liu, Z. and A. Davidson, *BAFF and selection of autoreactive B cells.* Trends Immunol, 2011. **32**(8): p. 388-94.

250. Thien, M., et al., *Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches.* Immunity, 2004. **20**(6): p. 785-98.

251. Ota, M., et al., *Regulation of the B cell receptor repertoire and self-reactivity by BAFF.* J Immunol, 2010. **185**(7): p. 4128-36.

252. Klasse, J. and J. Blomberg, *Patterns of antibodies to human immunodeficiency virus proteins in different subclasses of IgG.* J Infect Dis, 1987. **156**(6): p. 1026-30.

253. Sundqvist, V.A., et al., *Restricted IgG subclass responses to HTLV-III/LAV and to cytomegalovirus in patients with AIDS and lymphadenopathy syndrome.* J Infect Dis, 1986. **153**(5): p. 970-3.

254. Ljunggren, K., et al., *IgG subclass response to HIV in relation to antibody-dependent cellular cytotoxicity at different clinical stages.* Clin Exp Immunol, 1988. **73**(3): p. 343-7.

255. Chiodi, F., et al., *IgG subclass responses to a transmembrane protein (gp41) peptide in HIV infection.* J Immunol, 1989. **142**(11): p. 3809-14.

256. McDougal, J.S., et al., *Antibody response to human immunodeficiency virus in homosexual men. Relation of antibody specificity, titer, and isotype to clinical status, severity of immunodeficiency, and disease progression.* J Clin Invest, 1987. **80**(2): p. 316-24.

257. Khalife, J., et al., *Isotypic restriction of the antibody response to human immunodeficiency virus.* AIDS Res Hum Retroviruses, 1988. **4**(1): p. 3-9.

258. Broliden, P.A., et al., *Fine specificity of IgG subclass response to group antigens in HIV-1-infected patients.* Clin Exp Immunol, 1989. **76**(2): p. 216-21.

259. Scharf, O., et al., *Immunoglobulin G3 from polyclonal human immunodeficiency virus (HIV) immune globulin is more potent than other subclasses in neutralizing HIV type 1.* J Virol, 2001. **75**(14): p. 6558-65.

260. Mathiesen, T., et al., *Analysis of a subclass-restricted HIV-1 gp41 epitope by omission peptides.* Immunology, 1989. **67**(1): p. 1-7.

261. Kovacsovics-Bankowski, M., et al., *Isotypic distribution of HIV-1-specific antibodies in individuals from central Africa.* Viral Immunol, 1992. **5**(4): p. 243-8.

262. Rowland-Jones, S., *Dimers are a girl's best friend.* Nat Med, 1997. **3**(11): p. 1199-200.

263. Hirbod, T., K. Broliden, and R. Kaul, *Genital immunoglobulin A and HIV-1 protection: virus neutralization versus specificity.* Aids, 2008. **22**(17): p. 2401-2.

264. Hirbod, T., et al., *HIV-neutralizing immunoglobulin A and HIV-specific proliferation are independently associated with reduced HIV acquisition in Kenyan sex workers.* Aids, 2008. **22**(6): p. 727-35.

265. Tomaras, G.D. and B.F. Haynes, *HIV-1-specific antibody responses during acute and chronic HIV-1 infection.* Current opinion in HIV and AIDS, 2009. **4**(5): p. 373-379.

266. Janeway CA Jr, T.P., Walport M, et al., *Immunobiology: The Immune System in Health and Disease. 5th edition.* New York: Garland Science, 2001.

267. Bernasconi, N.L., E. Traggiai, and A. Lanzavecchia, *Maintenance of serological memory by polyclonal activation of human memory B cells.* Science, 2002. **298**(5601): p. 2199-202.

268. Havenar-Daughton, C., et al., *CXCL13 is a plasma biomarker of germinal center activity.* Proc Natl Acad Sci U S A, 2016. **113**(10): p. 2702-7.

269. Abel, K., et al., *Temporal and anatomic relationship between virus replication and cytokine gene expression after vaginal simian immunodeficiency virus infection.* J Virol, 2005. **79**(19): p. 12164-72.

270. Dalod, M., et al., *Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo.* J Exp Med, 2002. **195**(4): p. 517-28.

271. Norris, P.J., et al., *Elevations in IL-10, TNF-alpha, and IFN-gamma from the earliest point of HIV Type 1 infection.* AIDS Res Hum Retroviruses, 2006. **22**(8): p. 757-62.

272. Borrow, P. and N. Bhardwaj, *Innate immune responses in primary HIV-1 infection.* Curr Opin HIV AIDS, 2008. **3**(1): p. 36-44.

273. Hassan, A.S., et al., *A Stronger Innate Immune Response During Hyperacute HIV-1 Infection is associated with ACUTE retroviral syndrome.* Clin Infect Dis, 2021.

274. Neilson, J.R., et al., *Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya.* J Virol, 1999. **73**(5): p. 4393-403.

275. Connor, R.I., et al., *Change in coreceptor use correlates with disease progression in HIV-1--infected individuals.* J Exp Med, 1997. **185**(4): p. 621-8.

276. Mackay, F. and C.R. Mackay, *The role of BAFF in B-cell maturation, T-cell activation and autoimmunity.* Trends Immunol, 2002. **23**(3): p. 113-5.

277. Cagigi, A., et al., *Altered expression of the receptor-ligand pair CXCR5/CXCL13 in B cells during chronic HIV-1 infection.* Blood, 2008. **112**(12): p. 4401-10.

278. Cohen, K.W., et al., *HIV-1 single-stranded RNA induces CXCL13 secretion in human monocytes via TLR7 activation and plasmacytoid dendritic cell-derived type I IFN.* J Immunol, 2015. **194**(6): p. 2769-75.

279. Widney, D.P., et al., *Serum levels of the homeostatic B cell chemokine, CXCL13, are elevated during HIV infection.* J Interferon Cytokine Res, 2005. **25**(11): p. 702-6.

280. Ho, J., et al., *Two overrepresented B cell populations in HIV-infected individuals undergo apoptosis by different mechanisms.* Proc Natl Acad Sci U S A, 2006. **103**(51): p. 19436-41.

281. Ayouba, A., et al., *Development of a Sensitive and Specific Serological Assay Based on Luminex Technology for Detection of Antibodies to Zaire Ebola Virus.* Journal of clinical microbiology, 2016. **55**(1): p. 165-176.

282. Bozza, F.A., et al., *Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis.* Crit Care, 2007. **11**(2): p. R49.

283. Mariani, E., et al., *Simultaneous evaluation of circulating chemokine and cytokine profiles in elderly subjects by multiplex technology: relationship with zinc status.* Biogerontology, 2006. **7**(5-6): p. 449-59.

284. Njemini, R., et al., *Heat shock proteins and chemokine/cytokine secretion profile in ageing and inflammation.* Mech Ageing Dev, 2007. **128**(7-8): p. 450-4.

285. Rudolph, J.L., et al., *Chemokines are associated with delirium after cardiac surgery.* J Gerontol A Biol Sci Med Sci, 2008. **63**(2): p. 184-9.

286. Verrier, B., et al., *Exploiting Natural Cross-reactivity between Human Immunodeficiency Virus (HIV)-1 p17 Protein and Anti-gp41 2F5 Antibody to Induce HIV-1 Neutralizing Responses In Vivo.* Frontiers in Immunology, 2017. **8**(770).

287. Keating, S.M., et al., *HIV Antibody Level as a Marker of HIV Persistence and Low-Level Viral Replication.* The Journal of infectious diseases, 2017. **216**(1): p. 72-81.

288. Macpherson, A.J., et al., *The immune geography of IgA induction and function.* Mucosal Immunology, 2008. **1**(1): p. 11-22.

289. van Egmond, M., et al., *IgA and the IgA Fc receptor.* Trends Immunol, 2001. **22**(4): p. 205-11.

290. Woof, J.M. and M.A. Kerr, *IgA function--variations on a theme.* Immunology, 2004. **113**(2): p. 175-177.

291. Steffen, U., et al., *IgA subclasses have different effector functions associated with distinct glycosylation profiles.* Nature Communications, 2020. **11**(1): p. 120.

292. Müller, F., M. Moskophidis, and H. Schmitz, *Intrathecal synthesis of specific IgG in syphilitic patients with human immunodeficiency virus 1 infection.* J Neurol, 1988. **235**(4): p. 252-3.

293. Mergener, K., et al., *Immunoglobulin class- and subclass-specific HIV antibody detection in serum and CSF specimens by ELISA and Western blot.* Infection, 1987. **15**(5): p. 317-22.

294. Landais, E. and P.L. Moore, *Development of broadly neutralizing antibodies in HIV-1 infected elite neutralizers.* Retrovirology, 2018. **15**(1): p. 61.

295. Legler, D.F., et al., *B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5.* J Exp Med, 1998. **187**(4): p. 655-60.

296. Overbaugh, J. and L. Morris, *The Antibody Response against HIV-1.* Cold Spring Harb Perspect Med, 2012. **2**(1): p. a007039.

297. Mikell, I., et al., *Characteristics of the earliest cross-neutralizing antibody response to HIV-1.* PLoS Pathog, 2011. **7**(1): p. e1001251.

298. Tomaras, G.D., et al., *Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia.* J Virol, 2008. **82**(24): p. 12449-63.

299. Robinson, W.E., Jr., D.C. Montefiori, and W.M. Mitchell, *Antibody-dependent enhancement of human immunodeficiency virus type 1 infection.* Lancet, 1988. **1**(8589): p. 790-4.

300. Robinson, W.E., Jr., D.C. Montefiori, and W.M. Mitchell, *A human immunodeficiency virus type 1 (HIV-1) infection-enhancing factor in seropositive sera.* Biochem Biophys Res Commun, 1987. **149**(2): p. 693-9.

301. Robinson, W.E., *Mechanism for complement-mediated, antibody-dependent enhancement of human immunodeficiency virus type 1 infection in MT2 cells is enhanced entry through CD4, CD21, and CXCR4 chemokine receptors.* Viral Immunol, 2006. **19**(3): p. 434-47.

302. Janeway CA Jr, T.P., Walport M, et al. , *Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001. The structure of a typical antibody molecule. Available from: https://www.ncbi.nlm.nih.gov/books/NBK27144/.*

303. Stanfield, R.L. and I.A. Wilson, *Antibody Structure.* Microbiol Spectr, 2014. **2**(2).

304. Abdul k, A.H., Shiv Pillai, *Cellular and Molecular Immunology, Ninth Edition International Edition.* 2018.

305. Boesch, A.W., E.P. Brown, and M.E. Ackerman, *The role of Fc receptors in HIV prevention and therapy.* Immunol Rev, 2015. **268**(1): p. 296-310.

306. Ackerman, M.E., et al., *Natural variation in Fc glycosylation of HIV-specific antibodies impacts antiviral activity.* J Clin Invest, 2013. **123**(5): p. 2183-92.

307. Chung, A.W., et al., *Identification of antibody glycosylation structures that predict monoclonal antibody Fc-effector function.* Aids, 2014. **28**(17): p. 2523-30.

308. Chiu, M.L., et al., *Antibody Structure and Function: The Basis for Engineering Therapeutics.* Antibodies (Basel, Switzerland), 2019. **8**(4): p. 55.

309. Bournazos, S. and J.V. Ravetch, *Diversification of IgG effector functions.* Int Immunol, 2017. **29**(7): p. 303-310.

310. Mahan, A.E., et al., *Antigen-Specific Antibody Glycosylation Is Regulated via Vaccination.* PLoS Pathog, 2016. **12**(3): p. e1005456.

311. Aasa-Chapman, M.M., et al., *Detection of antibody-dependent complement-mediated inactivation of both autologous and heterologous virus in primary human immunodeficiency virus type 1 infection.* J Virol, 2005. **79**(5): p. 2823-30.

312. Huang, Y., et al., *Diversity of Antiviral IgG Effector Activities Observed in HIV-Infected and Vaccinated Subjects.* J Immunol, 2016. **197**(12): p. 4603-4612.

313. Gray, E.S., et al., *The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection.* J Virol, 2011. **85**(10): p. 4828-40.

314. Scheid, J.F., et al., *Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals.* Nature, 2009. **458**(7238): p. 636-40.

315. Wilson, P.C., et al., *Somatic hypermutation introduces insertions and deletions into immunoglobulin V genes.* J Exp Med, 1998. **187**(1): p. 59-70.

316. Poignard, P., et al., *Heterogeneity of envelope molecules expressed on primary human immunodeficiency virus type 1 particles as probed by the binding of neutralizing and nonneutralizing antibodies.* J Virol, 2003. **77**(1): p. 353-65.

317. Crooks, E.T., et al., *A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120.* Virology, 2007. **366**(2): p. 245-62.

318. Burton, D.R., et al., *A Blueprint for HIV Vaccine Discovery.* Cell Host Microbe, 2012. **12**(4): p. 396-407.

319. Burton, D.R. and J.R. Mascola, *Antibody responses to envelope glycoproteins in HIV-1 infection.* Nat Immunol, 2015. **16**(6): p. 571-6.

320. Kong, R., et al., *Fusion peptide of HIV-1 as a site of vulnerability to neutralizing antibody.* Science, 2016. **352**(6287): p. 828-33.

321. Ditse, Z., et al., *HIV-1 Subtype C-Infected Children with Exceptional Neutralization Breadth Exhibit Polyclonal Responses Targeting Known Epitopes.* J Virol, 2018. **92**(17).

322. Hessell, A.J., et al., *Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers.* PLoS Pathog, 2009. **5**(5): p. e1000433.

323. Ruprecht, R.M., et al., *Protection of neonatal macaques against experimental SHIV infection by human neutralizing monoclonal antibodies.* Transfus Clin Biol, 2001. **8**(4): p. 350-8.

324. Calarese, D.A., et al., *Antibody domain exchange is an immunological solution to carbohydrate cluster recognition.* Science, 2003. **300**(5628): p. 2065-71.

325. Ofek G1, G.F., Schief WR, Skinner J, Baker D, Wyatt R, Kwong PD., *Elicitation of structure-specific antibodies by epitope scaffolds.* Proc Natl Acad Sci U S A., 2010. **107(42):17880-7. doi: 10.1073/pnas.1004728107. Epub 2010 Sep 27.**

326. Correia, B.E., et al., *Computational protein design using flexible backbone remodeling and resurfacing: case studies in structure-based antigen design.* J Mol Biol, 2011. **405**(1): p. 284-97.

327. Haynes BF1, M.M., Verkoczy L, Kelsoe G, Alam SM., *Antibody polyspecificity and neutralization of HIV-1: a hypothesis.* Hum antibodies, 2005. **14(3-4):59-67.**

328. Rusert, P., et al., *Determinants of HIV-1 broadly neutralizing antibody induction.* Nat Med, 2016. **22**(11): p. 1260-1267.

329. Moore, P.L., C. Williamson, and L. Morris, *Virological features associated with the development of broadly neutralizing antibodies to HIV-1.* Trends Microbiol, 2015. **23**(4): p. 204-11.

330. McElrath, M.J. and B.F. Haynes, *Induction of immunity to human immunodeficiency virus type-1 by vaccination.* Immunity, 2010. **33**(4): p. 542-54.

331. Petrovas, C., et al., *CD4 T follicular helper cell dynamics during SIV infection.* J Clin Invest, 2012. **122**(9): p. 3281-94.

332. Rademeyer, C., et al., *Genetic characteristics of HIV-1 subtype C envelopes inducing cross-neutralizing antibodies.* Virology, 2007. **368**(1): p. 172-81.

333. Seaman, M.S., et al., *Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies.* J Virol, 2010. **84**(3): p. 1439-52.

334. Ana-Sosa-Batiz, F., et al., *HIV-specific antibody-dependent phagocytosis matures during HIV infection.* Immunol Cell Biol, 2014. **92**(8): p. 679-87.

335. Tsachouridou, O., et al., *Deficient Phagocytosis Among HIV-1 Infected Adults Over Time Even in HAART Setting.* Curr HIV Res, 2017. **15**(4): p. 285-290.

336. Dugast, A.-S., et al., *Decreased Fc receptor expression on innate immune cells is associated with impaired antibody-mediated cellular phagocytic activity in chronically HIV-1 infected individuals.* Virology, 2011. **415**(2): p. 160-167.

337. Dugast, A.S., et al., *Independent evolution of Fc- and Fab-mediated HIV-1-specific antiviral antibody activity following acute infection.* Eur J Immunol, 2014. **44**(10): p. 2925-37.

338. Youd, M.E., A.R. Ferguson, and R.B. Corley, *Synergistic roles of IgM and complement in antigen trapping and follicular localization.* Eur J Immunol, 2002. **32**(8): p. 2328-37.

339. Heyman, B., L. Pilstrom, and M.J. Shulman, *Complement activation is required for IgM-mediated enhancement of the antibody response.* J Exp Med, 1988. **167**(6): p. 1999-2004.

340. Prabhu, V.M., et al., *Monocyte Based Correlates of Immune Activation and Viremia in HIV-Infected Long-Term Non-Progressors.* Frontiers in immunology, 2019. **10**: p. 2849-2849.

341. Gartner, S., et al., *The role of mononuclear phagocytes in HTLV-III/LAV infection.* Science, 1986. **233**(4760): p. 215-9.

342. Sips, M., et al., *Fc receptor-mediated phagocytosis in tissues as a potent mechanism for preventive and therapeutic HIV vaccine strategies.* Mucosal immunology, 2016. **9**(6): p. 1584-1595.

343. Chung, A.W., et al., *Polyfunctional Fc-effector profiles mediated by IgG subclass selection distinguish RV144 and VAX003 vaccines.* Sci Transl Med, 2014. **6**(228): p. 228ra38.

344. Sather, D.N., et al., *Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection.* J Virol, 2009. **83**(2): p. 757-69.

345. Doria-Rose, N.A., et al., *Breadth of human immunodeficiency virus-specific neutralizing activity in sera: clustering analysis and association with clinical variables.* J Virol, 2010. **84**(3): p. 1631-6.

346. Plotkin, S.A., *Correlates of protection induced by vaccination.* Clin Vaccine Immunol, 2010. **17**(7): p. 1055-65.

347. Martinez, V., et al., *Combination of HIV-1-Specific CD4 Th1 Cell Responses and IgG2 Antibodies Is the Best Predictor for Persistence of Long-Term Nonprogression.* The Journal of Infectious Diseases, 2005. **191**(12): p. 2053-2063.

348. Ngo-Giang-Huong, N., et al., *HIV type 1-specific IgG2 antibodies: markers of helper T cell type 1 response and prognostic marker of long-term nonprogression.* AIDS Res Hum Retroviruses, 2001. **17**(15): p. 1435-46.

349. Smalls-Mantey, A., et al., *Antibody-dependent cellular cytotoxicity against primary HIV-infected CD4+ T cells is directly associated with the magnitude of surface IgG binding.* J Virol, 2012. **86**(16): p. 8672-80.

350. Streeck, H., et al., *Harnessing $CD4^+$ T cell responses in HIV vaccine development.* Nat Med, 2013. **19**(2): p. 143-9.

351. Kinoshita, K. and T. Honjo, *Linking class-switch recombination with somatic hypermutation.* Nat Rev Mol Cell Biol, 2001. **2**(7): p. 493-503.

352. Tudor, D., et al., *Isotype modulates epitope specificity, affinity, and antiviral activities of anti-HIV-1 human broadly neutralizing 2F5 antibody.* Proc Natl Acad Sci U S A, 2012. **109**(31): p. 12680-5.

353. Janda, A., et al., *Ig Constant Region Effects on Variable Region Structure and Function.* Front Microbiol, 2016. **7**: p. 22.

354. Moir, S. and A.S. Fauci, *Insights into B cells and HIV-specific B-cell responses in HIV-infected individuals.* Immunol Rev, 2013. **254**(1): p. 207-24.

355. Cagigi, A., et al., *B cell immunopathology during HIV-1 infection: lessons to learn for HIV-1 vaccine design.* Vaccine, 2008. **26**(24): p. 3016-25.

356. Negera, E., et al., *Increased activated memory B-cells in the peripheral blood of patients with erythema nodosum leprosum reactions.* PLoS neglected tropical diseases, 2017. **11**(12): p. e0006121-e0006121.

357. Raziorrouh, B., et al., *Virus-Specific CD4+ T Cells Have Functional and Phenotypic Characteristics of Follicular T-Helper Cells in Patients With Acute and Chronic HCV Infections.* Gastroenterology, 2016. **150**(3): p. 696-706.e3.

358. Lu, J., et al., *Expansion of circulating T follicular helper cells is associated with disease progression in HIV-infected individuals.* J Infect Public Health, 2018. **11**(5): p. 685-690.

359. Thorarinsdottir, K., et al., *CD21 -/low B cells: A Snapshot of a Unique B Cell Subset in Health and Disease.* Scand J Immunol, 2015. **82**(3): p. 254-61.

360. Moir, S., et al., *Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals.* J Exp Med, 2008. **205**(8): p. 1797-805.

361. Martin-Gayo, E., et al., *Circulating CXCR5(+)CXCR3(+)PD-1(lo) Tfh-like cells in HIV-1 controllers with neutralizing antibody breadth.* JCI insight, 2017. **2**(2): p. e89574-e89574.

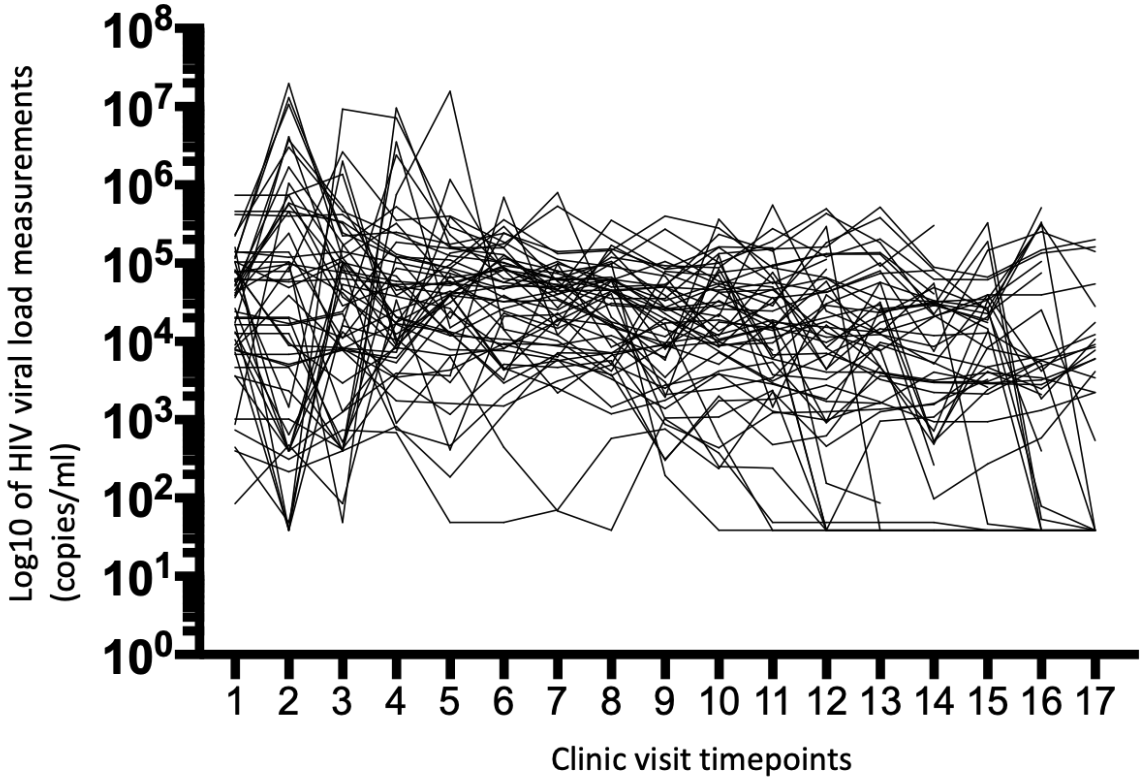
362. Mattapallil, J.J., et al., *Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection.* Nature, 2005. **434**(7037): p. 1093-7.

363. Li, Q., et al., *Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells.* Nature, 2005. **434**(7037): p. 1148-52.

364. Widney, D.P., et al., *Serum levels of the homeostatic B cell chemokine, CXCL13, are elevated during HIV infection.* J Interferon Cytokine Res, 2005. **25**(11): p. 702-6.

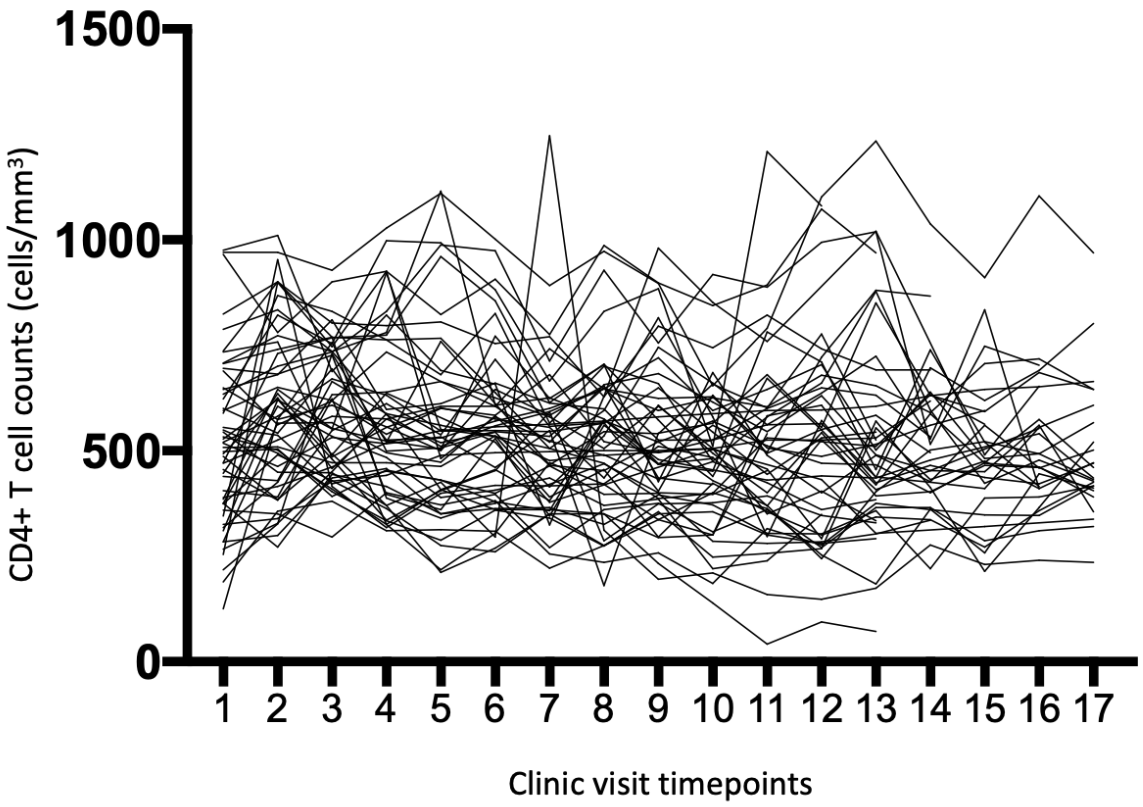
Appendices

1. The trends of viral load measurements over the course of HIV disease



Each trend line represents a study participant's Log10 HIV viral load measurements in the course of HIV disease. The y-axis represents Log10 HIV viral load measurements in copies/ml, while the x-axis represents quarterly clinic visit time points in the cohort. GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to generate the line graphs (Line graphs (Spaghetti plots)).

2. The trends of CD4+ T cell counts over the course of HIV disease



Each trend line represents a study participant's CD4+ T cell counts in the course of HIV disease. The y-axis represents CD4+ T cell counts in cells/mm^3 while the x-axis represents quarterly clinic visit time points in the cohort. GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to generate the line graphs (Line graphs (Spaghetti plots)).

3. Ethical approval for protocol C participants

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) 2722541, 2713349, 0722-205901,0733-400003, Fax: (254) (020) 2720030
Email: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **July 03, 2020**

TO: **DR. EDUARD SANDERS,**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DEPUTY DIRECTOR, CGMR-C,**
KILIFI.

Dear Sir,

RE: **PROTOCOL NO. SSC 1027 *(REQUEST FOR ANNUAL RENEWAL)*: A PROSPECTIVE, OBSERVATIONAL, MULTICENTRE STUDY TO EVALUATE LABORATORY, CLINICAL, IMMUNOLOGIC AND VIRAL MARKERS OF DISEASE PROGRESSION IN RECENTLY HIV INFECTED VOLUNTEERS**

Thank you for the continuing review report for the period **June 03, 2019** to **May 31, 2020.**

The Expedited Review Team acknowledges receipt of the following documents:

1. Continuing Review Report
2. SERU annual approval letter dated 09 July 2019
3. Currently Approved Protocol vs 6.1 dated 24 April 2017
4. Deviation Report

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **July 18, 2020** through to **July 17, 2021**. Please note that authorization to conduct this study will automatically expire on **July 17, 2021**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **June 05, 2021.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

4. Informed consent form for Protocol C volunteer enrolment

SSC#_1027 (revision /amended: 24th April 2017) _Acute_HIV_Inf_Study_ESanders

Study ID 00-C21-|__|__|__|__|

INFORMED CONSENT FOR VOLUNTEER ENROLLMENT
IN MULTI-CENTRE STUDY OF ACUTE HIV INFECTIONS

TITLE: A PROSPECTIVE, OBSERVATIONAL, MULTI-CENTER STUDY TO EVALUATE LABORATORY, CLINICAL, IMMUNOLOGIC AND VIRAL MARKERS OF DISEASE PROGRESSION IN RECENTLY HIV-INFECTED VOLUNTEERS

Rationale for this Study

Over 33 million people worldwide are currently infected with human immunodeficiency virus (HIV), the virus that causes AIDS. New people are being infected every day. Only a limited amount of information is available on how HIV infects people's immune cells and how our immune systems react to this infection. This is especially true for HIV infection in Africa. By participating in this study, you can help expand the field of knowledge on how HIV affects Africans and how African strains of HIV are controlled by the body. In addition, it is hoped that this information may help scientists to design a vaccine to prevent HIV, especially in Africa and other areas of the world most affected by this epidemic. Hopefully the information we collect in this study will also help improve clinical care of Africans infected by HIV.

You are being asked to participate in this research study because you have become infected with the HIV virus just prior to entering the study. Enrollment of new volunteers into the study stopped at the end of 2011. Volunteers currently enrolled in this study are being requested to participate in a new follow-up regimen will look at how HIV infection progresses and how the cells in your body are trying to fight HIV- infection overtime. In order to get this information we will continue to assess your health regularly and collect blood samples and analyze them but the timing and schedule of visits may change slightly for you. Performing these tests will also be helpful in preparing KEMRI for future, larger, HIV prevention vaccine trials. Additionally, this research study will refer you for treatment, care and support if you need it. The new follow-up regimen applies to all currently enrolled volunteers and is explained below

Background

The International AIDS Vaccine Initiative (IAVI), the Sponsor of this study, is an international, scientific, non-profit organization, whose mission it is to ensure the development of a safe and effective, preventive vaccine against HIV and to ensure that if such a vaccine is found, that it becomes available to those that need it most. IAVI does not provide treatment for HIV but will make every attempt to ensure that you are referred for treatment.

Duration of the Study

The length of your participation in this study is dependent upon:

- Your willingness and ability to participate
- How long you have been infected with HIV

APPROVED (11Sept 2017)

- When you enrolled in the study
- Whether your participation can help the researchers answer scientific questions related to this study

Individual participation may vary, but it will not exceed 10 years. If you have been HIV-infected for less than 1 year, you will have study visits every 3 months for the first year after you became infected. After that you will have visits every 6 months until the study ends. If you have been HIV-infected for more than 1 year, you will have visits every 6 months until the study ends. Additional visits may be scheduled if you need care, treatment, and counseling to get results of your laboratory tests.

Your Participation Is Voluntary

This consent form gives information about the study that will be discussed with you and read to you if you are unable to read, in a language you are comfortable with. If you agree to participate in the new follow up regime, you will sign your name or make your mark on 2 copies of this form confirming that you agree to take part and you are doing so voluntarily; one copy is for you to keep and one will be kept at KEMRI. If you do not wish to keep your copy, it will be kept at the site in a secure place for you. If you wish to have a person who is not part of the study team help you understand the study, they may be present with you during the consent process and they will be required to witness your consent.

It is important that you know the following:
- Your participation is voluntary.
- You may decide to stop being part of the study at any time.
- You will not lose any rights or benefits if you do not participate.

STUDY PROCEDURES

If you agree to participate in the new follow up schedule for this study and sign or mark this form, the following procedures will take place for first 3 years of study visits after enrolment:

At every visit (after enrolment though month 36):

You will be asked questions regarding your health, your sexual activities and any medications you are taking, and you will have routine medical examinations. For women this includes a pelvic examination. For individuals who report receptive anal sex, we will also offer an examination by proctoscopy to identify anal ulcers or proctitis.

If you are referred for treatment, you will be asked questions regarding whether you followed up on the referral and the reasons you may not have gone for the referral visit.

Up to 10 tablespoons (100 mls) of blood will be taken at each visit. Some of this blood is required;
- To determine your general health and to assist with your treatment (such as how much virus there is in your blood and the number of immune T cells that are in your blood).

You will be informed of your blood cell count and the number of CD4 and CD8 T cells in your blood.

- To be stored for possible future testing that will help in research in AIDS vaccines and genetic testing related to your immune system to better understand the transmission and progression of HIV.
- To be tested again to ensure that tests in the laboratory are always done correctly and carefully. If your test results are not clear, as sometimes happens, some of this stored blood may be used to test your blood again, using special tests.

Your study doctor may request additional samples for your treatment. You will only be given the results of the tests that assist with your treatment. General information about what these tests have taught us about HIV will be shared with the volunteers as a group, the Ethics Committee, and with the Investigators.

At your Month 12 visit, and annually thereafter, your blood will be tested for syphilis. You will be provided these results and provided treatment if needed.

You will be offered counselling on how to deal with problems related to your HIV infection, what it means for your sexual partners and family members, and how to avoid transmitting HIV to others in future. If you wish, your partner and/or family members can have counselling with you.

Women will have a urine sample to test for pregnancy. You can participate in this study if you are or become pregnant.

You are allowed to participate in other research studies at the same time you are in this study. If you are already involved with on-going studies with these investigators, we will use information we have already collected from you rather than collect new information, provided you give us permission to do this. The doctor will also ask you questions about other studies to ensure that the amount of blood taken will be within limits to protect your health.

Study Visits after 3.5 years

If you would like to continue in the study after 3.5 years (42 months) of study visits from the estimated date you acquired HIV, the researchers will assign you to 1 of 2 follow-up tracks. Assignment is based on certain test results throughout the study. The tests indicate how your immune system is responding to the HIV virus.

Track 1 (Standard Visit)

Every 6-monthly study visit will be the same as before—the procedures will not change from those done for the first 3.5 years. As before, up to 10 tablespoons [or local equivalent of 100mL] of blood will continue to be drawn from your arm at every visit. Your blood will be collected and stored to determine your general health and to study how your immune cells are responding to the virus.

Track 2 (Clinical Visit)
Every 6-monthly visit will be similar to your previous visits except that less clinical information will be collected and up to 2 tablespoons (20 mls) of blood will be drawn from your arm at every visit. Your blood will be collected primarily to determine your general health and provide test results that may be important for your clinical care. A small amount of the blood collected will be stored for research purposes and may be tested in the future to determine how your body is responding to the virus.

What if I become pregnant?
You can continue in the study if you become pregnant, and you are provided ARVs for the prevention of mother-to-child-transmission (PMTCT) of HIV. Fewer blood samples for this study will be drawn while you are pregnant and for the 3 months after you deliver. Your study doctor will discuss which samples may be omitted. If you become pregnant, you will also be counseled and referred for appropriate prenatal care.

What if I start taking ARVs?
If you are not pregnant and you start taking continuous long term ARVs, you will be released from the study if you have access to regular HIV care elsewhere in the community. If you start taking continuous long term ARVs and you do NOT have access to regular HIV care, you may complete your follow-up visits in Track 2 (Clinical Visit), if you choose to continue in the study.

Stored blood
Your stored blood may be sent to other expert laboratories for additional tests. It is not known at this time to which laboratories these samples will be sent or for how long the samples will be stored. However, the samples may be stored for an additional 10 years after they study closure at the KEMRI-CGMRC and at IAVI Human Immunology Laboratory in London. Your blood that is stored will only be labelled by a study specific number and not with your name to ensure that no one, other than people running the study knows who you are. No other tests will be performed without the approval of the ethics committee. Any future work not mentioned in the initial protocol will require additional approval from the relevant authorities. You will not receive the results of tests done at special laboratories as they are research tests and are not linked to your name and do not affect your care.

If you were a volunteer in protocol SSC#894_revised (the cohort study in Kilifi or Mtwapa), your stored blood from that study may be used to determine whether there was any HIV virus present in your blood before you became positive on the antibody test. In addition, data from this previous study, such as your CD4 counts and other study data may be used in this study to provide background information on you.

Some of the stored blood may be used for genetic testing. The genetic testing is being done to see if different types of immune responses may be related to genetic differences in people. The testing will be done in a laboratory using the stored blood identified only by a sample number. Your name will not appear with the sample. The results of the genetic testing will appear only in confidential study-related documents (and not in your medical record) nor will you be told the results of the testing.

Risks and /or Discomforts

There may be some risk involved in drawing blood for the tests which sometimes can cause pain and bruising where the needle goes into your arm. When blood is being drawn, you may feel dizzy or faint but this is not common.

You may become embarrassed, worried, or anxious when discussing your sexual practices, including ways to prevent transmitting HIV to others. Knowing that you have HIV or other infections passed during sex could make you worried or anxious. A trained counselor will help you talk about these feelings or questions you may have.

We will ensure that your privacy is protected during and after the study. Your participation in this study may be associated with discrimination, particularly if you tell family, friends or others about your participation in this study. It is also possible that others may learn of your participation in this study and work out that you have been infected with the HIV virus. Because of this, others may treat you unfairly or discriminate against you. There are several research studies being conducted at this research centre in HIV-infected and uninfected people which decreases the chance of this happening.

Benefits

You may benefit by taking part in the study from receiving counseling, regular examinations and your CD4 count provided to you or your physician with your permission. You will be referred for appropriate care for illnesses and HIV (including ARVs and medications to prevent infections in people who are infected with HIV) if it cannot be provided at the clinic. You or others may benefit in the future from information learned in this study. If you acquire a sexually transmitted disease, you will be examined and treated in the clinic; if treatment is not available here, you will be referred for care elsewhere. You may get some personal satisfaction from being part of research on HIV. If you are a woman, and you become pregnant, you will be referred for prenatal care and for PMTCT.

Injuries

We do not expect you to suffer any injury as a result of participating in this study, but if you do, KEMRI personnel will give you the necessary treatment for your injuries including emergency treatment without charge. If we cannot manage the injury at our site, you will be told where you can get additional treatment for your injuries. You will not have to pay for treatment that is needed due to a direct result of study participation. There is no program for monetary compensation or other forms of compensation for such injuries. You do not give up any legal rights by signing this consent form.

Referral for Care and Treatment

Comprehensive HIV care will be provided to you at the KEMRI-Mtwapa clinic or Kilifi District Hospital Comprehensive Care and Research Clinic. For chronic illnesses, or other diseases not related to HIV, you will be referred to any of the Government clinics of your choice.

Costs to You

There is no cost to you for being in the study. However volunteers will be compensated for costs to cover travel expenses to the study site at Ksh 500 for each scheduled visit and any inconvenience caused due to study participation.

Circumstances for Withdrawal from the Study

You may be removed from the study without your consent for the following reasons:

- **You are not able to attend study visits or complete the study procedures**
- **If the study is stopped or cancelled**
- **Other administrative reasons**

Alternatives to Participation

The study staff will inform you if there are other HIV research studies taking place in your community and if there are other facilities where you can go for HIV counseling, care and support.

New Information

You will be told of any new information gained during the course of the study. You will be told when the results of the study may be available, and how to learn about them.

Supervision of the Study

This study will be approved by the site's Ethics Review Committee - a group that looks out for the well-being of all study participants to ensure that the rights of volunteers are protected. In addition, the conduct of the study will be supervised by a group of experts called the Steering Committee. All information collected will be regularly checked by independent study monitors.

Confidentiality

Your participation in the study, all information collected about you as well as all results of laboratory tests will be private and not available to others outside of the people listed below. You will have your own special identity number known only to you and the clinic staff. Apart from the study team members that you meet, other staff from National or international government bodies that ensure correct conduct of research, members of the Ethics Committee, study monitors, auditors, inspectors, and representatives of the Sponsor (IAVI) will check the records to make sure that the study was conducted properly. They are required to respect your confidentiality. Stored blood and clinical data will only be labeled with your study-specific number and not with your name. This is to ensure that no one, other than people running the study, knows that the samples belong to you. Any samples and study data that are shared with expert laboratories will only be identified by this study number. These laboratories will never have access to your personal details. Your identity will not be disclosed in any publication or presentation of this study. Your blood specimens will only be identified by your study number. Any documents containing your name will be locked away in a secure place.

Contacting you at home
Once you enroll in the study, we will offer 3 strategies to improve the research participation:

Home visits:
If you grant permission, clinic staff will make an appointment to visit you once at your home. During this visit, they will update your tracing locator form with landmarks in the vicinity of your home and take a GPS-way point. A GPS machine looks like a big mobile telephone and can take coordinates of any location with a precision of about 5 meters. These coordinates will be used by KEMRI-UW staff only if we need to trace you for an abnormal lab results or missed visit. We believe this technology will help us trace you more discretely, since tracing staff will need to ask fewer questions to locate your residence. Study staff will only come to your home with your permission.

SMS-reminder message:
If you provide us with a mobile phone number, we will send you a reminder message for your clinic appointment 1 day in advance. If you do not want to receive such a message, please let us know either now or at any time in the future.

Fingerprint scan:

To help us keep track of who is enrolled in our clinic, we will ask you to put your right index finger on a small machine that can scan your finger print. This will be done at each clinic visit and will take less than 5 seconds. This scan will translate into a unique identification number that will be added to your study ID card, and will be accessible only to our staff and to staff of the KAVI Kangemi research in Nairobi. If you are in Nairobi and need emergency care, you are welcome at the Kangemi clinic. Your finger scan will let the Kangemi clinic staffs know that you are enrolled in the KEMRI clinics in Mtwapa and Kilifi.

If you don't want to have your fingerprint taken, please let us know – you are free to refuse.

Do you agree to have your stored blood sent to other expert laboratories for possible future testing to help in research for AIDS vaccines? No additional tests will be performed without the approval of the Ethics Committee

☐ Yes ☐ No If yes, pls complete the sample storage form

Do you agree to be visited at home?

☐ Yes ☐ No

Do you agree to receive SMS reminder message for your clinic appointment?

☐ Yes ☐ No

Do you agree to have your finger scanned each time you visit the clinic

☐ Yes ☐ No

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Who should I contact with any questions about the study?

The coordinators are:
Dr. Eduard Sanders. If you have any questions you can reach him at KEMRI (telephone: 0725 242233).

If you have a medical problem related to your participation, please contact The Director KEMRI (telephone: 0725 242233).

Nurse/Counsellors are available at KEMRI Kilifi tel no. 0725 242233 or KEMRI Mtwapa 0722 206418

If you have a question about the ethics of this investigation you can contact Community Liaison Manager, (0725 242233, or 0723 342780) at KEMRI, or

The Head, KEMRI Scientific and Ethics Review Unit, P. O. BOX 54840-00200, Nairobi. Telephone: 0717 719477 or 0776399979. Email address: seru@kemri.org

RESEARCH PARTICIPANT'S STATEMENT

This study has been explained to me, and I voluntarily consent to participate. I have had an opportunity to ask questions. I understand that future questions I may have about the research will be answered by one of the investigators. I will receive a copy of this consent form.

Name of research participant _____

Signature of research participant _____ Date _____

Name and signature of witness

Name _____Signature _____Date _____

(If research participant was not able to read and understand the informed consent document)

Name and Signature of Person Obtaining Consent

Name _____Signature _____Date _____

Copies to: investigator, research participant

Study ID 00-C21-|__|__|__|__|

SAMPLE STORAGE AND SHIPPING CONSENT FORM SIGNATURE PAGE

I, (name of volunteer) _____
agree that after the tests needed for the study are done, the remaining blood taken from me may be stored for an additional 10 years after study closure. I understand that a number, rather than my name, will be used to label this blood so it is confidential. The stored blood may be used in the future for quality control purposes and other tests related to KEMRI ERC approved HIV vaccine research and development.

I also agree that my blood will be sent to external, national or international laboratories including the Human Immunology Laboratory of IAVI located in London. All of these tests will be for HIV vaccine research only.

I understand that some of the blood drawn as part of this study may be used for genetic testing. The genetic testing is being done to see if different types of immune responses may be related to genetic differences in people. The testing will be done in a laboratory using the stored blood identified only by a sample number. My name will not appear with the sample. The results of the genetic testing will appear only in confidential study-related documents (and not in my medical record) nor will I be told the results of the testing.

New genetic research tests to help understand if a vaccine might work may be done on stored samples in the future. If new information about HIV infection becomes known through this research, the information will be shared with me and the ethics committee.

Participant Signature: _____

Name: _____ Date: _____

Person obtaining consent:

Signature of person obtaining consent: _____

Name: _____ Date: _____

Impartial witness (if volunteer is illiterate):
I sign here as a witness to the consent process. I have participated in the discussion and witnessed the volunteer's consent to study participation:

Impartial witness Signature _____

Name: _____Date: _____

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5. Ethical approval for HIV-naïve volunteer enrolment

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel:(254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website.www.kemri.org

KEMRI/RES/7/3/1

February 27, 2019

TO: FRANCIS NDUNGU,
PRINCIPAL INVESTIGATOR.

THROUGH: THE DIRECTOR, CGMR-C,
KILIFI.

Received: 7/3/19

DIRECTOR
CENTRE FOR GEOGRAPHIC MEDICINE RESEARCH, COAST

Dear Sir,

RE: KEMRI/SERU/CGMR-C/022/3149 (*REQUEST FOR ANNUAL RENEWAL AND PROTOCOL DEVIATION*): SYSTEMS IMMUNOLOGY STUDIES OF *PLASMODIUM FACIPARUM* MALARIA SUSCEPTIBILITY IN KILIFI COUNTY

Thank you for the continuing review report for the period February 16, 2018 to February 05, 2019.

The Committee noted that a protocol deviation form has been submitted as the request for annual renewal was done after the date of submission required. Measures taken to address deviation are adequate.

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **March 15, 2019** through to **March 14, 2020**. Please note that authorization to conduct this study will automatically expire on **March 14, 2020**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **January 31, 2020.**

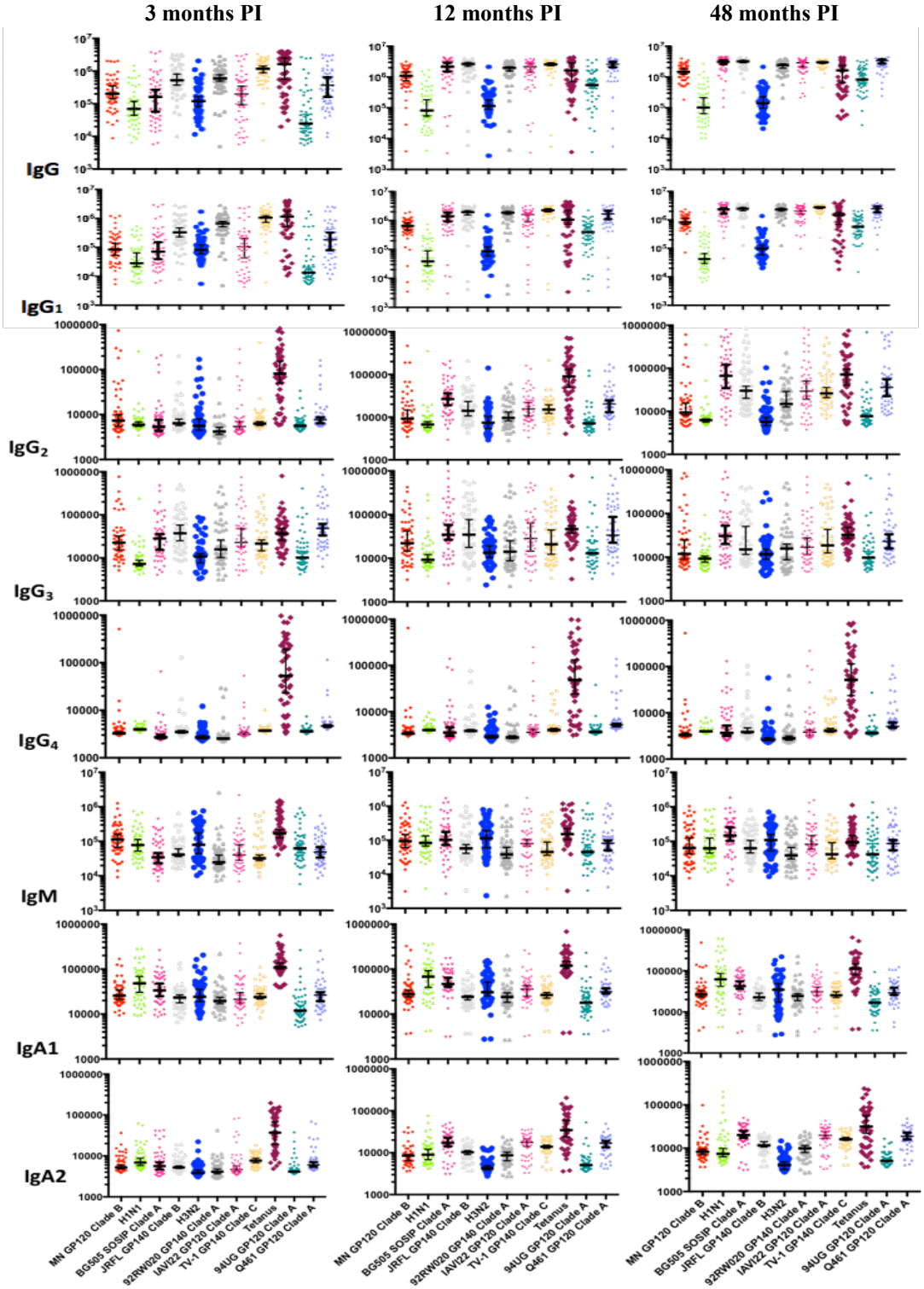
You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

Yours faithfully,

ENOCK KEBENEI
ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

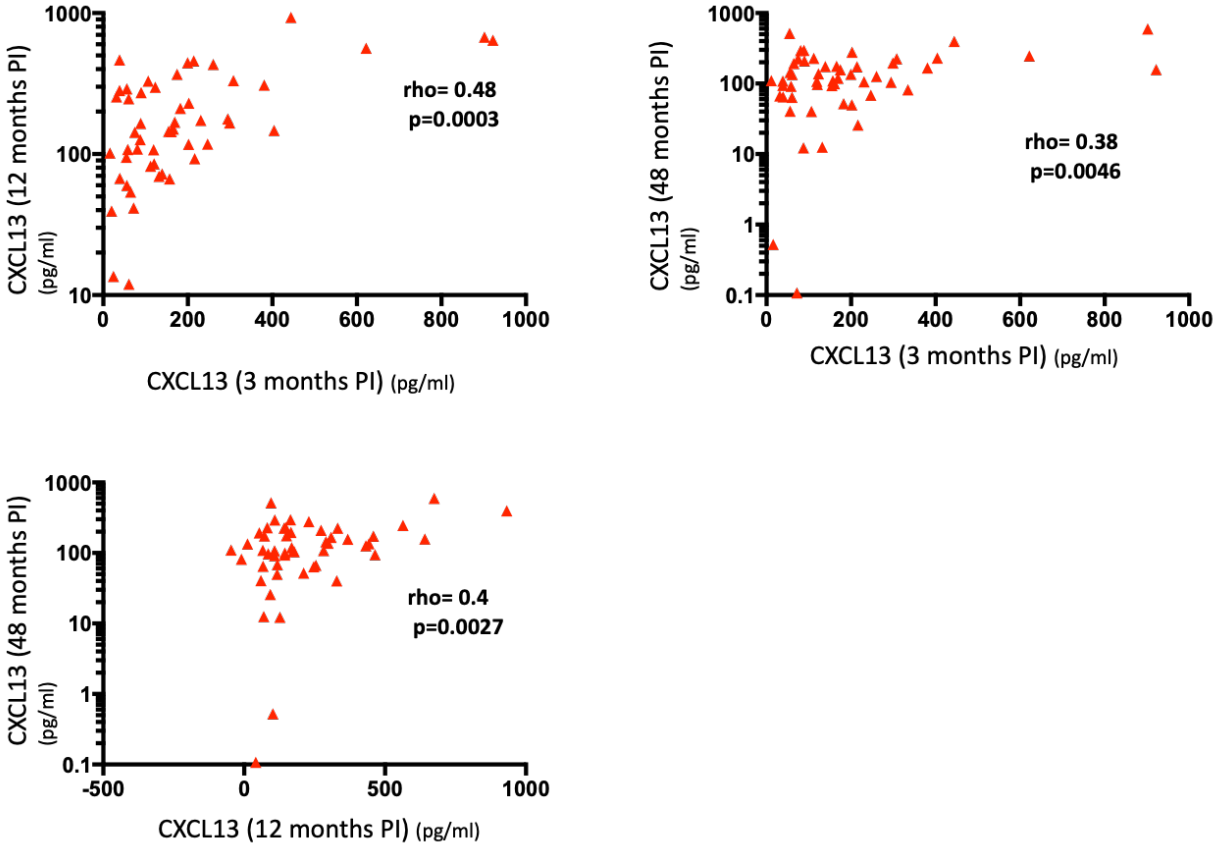
KEMRI Centre for Geographic Medicine Research - Coast
RECEIVED
07 MAR 2019
Tel: 07[illegible]
P.O. Box 428, Kilifi

6. The dynamics of HIV-specific antibody isotypes and subclasses levels as detected by the Luminex assay.



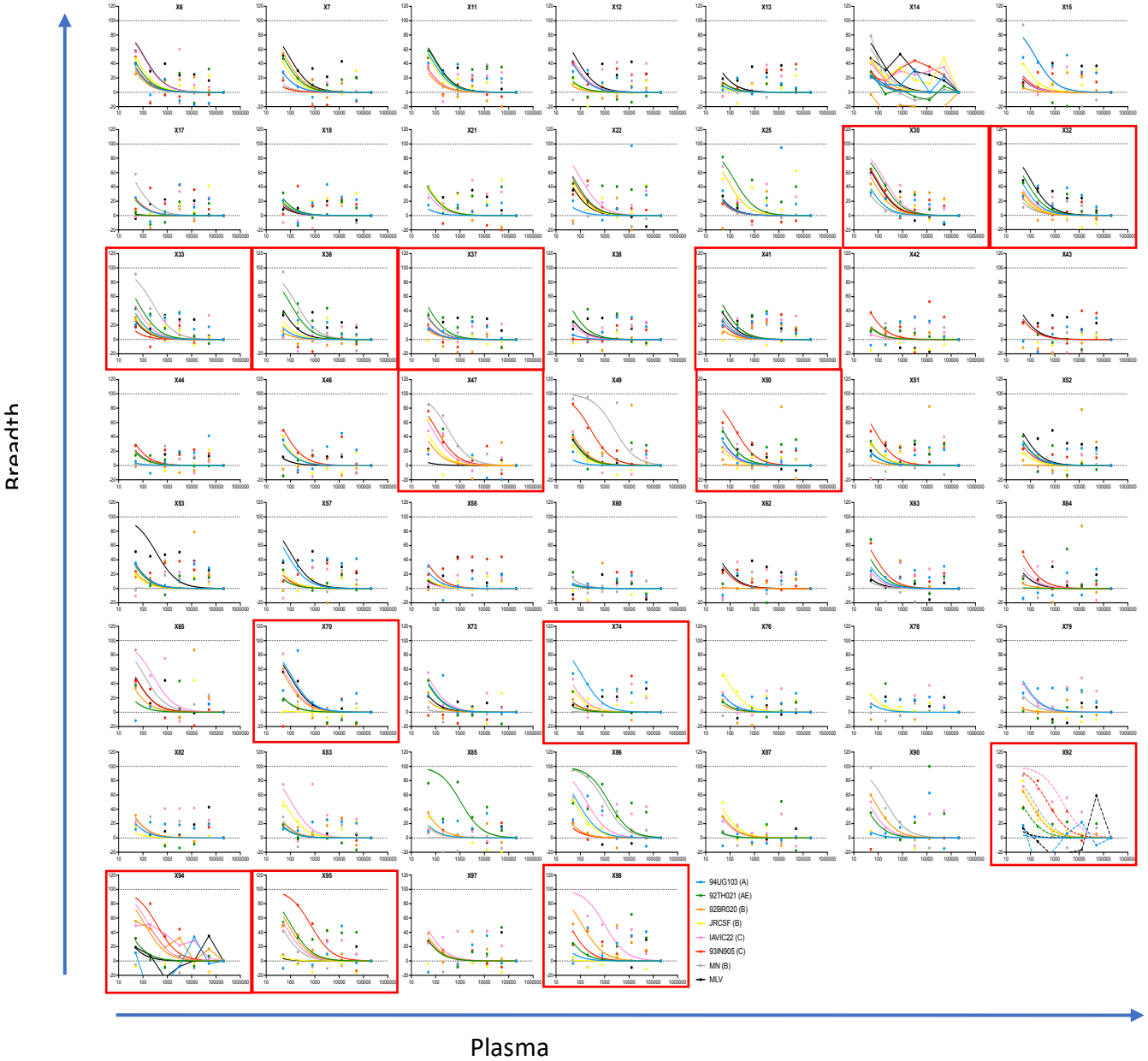
Eight HIV antigens spanning across clades A, B and C were used to detect HIV-specific responses while flu and tetanus antigens were used as controls. The median concentration for each sub-group and IQR values are shown. The y-axis represents mean fluorescence intensity (MFI) values; the x-axis represents different antigens used. PI represents post-HIV infection. Test, Kruskal-Wallis.

7. Correlation between the levels of CXCL13 in the course of HIV



Plasma CXCL13 levels positively correlated at 3-, 12- and 48 months post-HIV infection.

8. The neutralisation curves for the plasma samples tested for the development of breadth



Each curve represents a study participant as labelled at the top of the curve. Each pseudovirus is colour coded, and a key is provided to describe each colour. Only 15/53 individuals generated antibodies with breadth. The x-axis represents plasma dilutions while all the y-axis represents the breadth of each plasma sample tested. Any plasma sample with a breadth of >0 has been marked with a red box. Test; nonlinear regression- inhibitor versus response (3 parameters). GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to analyse the data.

9. List of reagents

11-0049-42	FITC anti-human CD4
17-9185-42	CD185 (CXCR5) Monoclonal Antibody (MU5UBEE), APC
	Anti-Human CD279 (PD-1) PE
12-2799-42	25
304133	Brilliant Violet 605™ anti-human CD45RA
353234	Brilliant Violet 650™ anti-human CD197 (CCR7)
46-9948-42	CD278 (ICOS) Monoclonal Antibody (ISA-3), PerCP-
	eFluor 710
348220	Brilliant Violet 510™ anti-human IgD, Clone IA6-2
	PE/Cy7 anti-human CD134
350012	(OX40)
12-0199-42	Anti-Human CD19 PE 100 tests
302234	Brilliant Violet 421™ anti-human CD19
302834	Brilliant Violet 711™ anti-human CD27, Clone O323
560179	Mouse Anti-Human CD8 APC-H7 (SK1 Clone) Monoclonal
	Antibody
302638	Brilliant Violet 785™ anti-human CD25
	Zombie Aqua™ Fixable Viability
423101	Kit
557917	Alexa Fluor® 700 Mouse Anti-Human CD3
344602	Purified anti-human CD4

4401	Cluster Tube System, 96-Well, Individual 1.2ml Tubes, Polypropylene, Non-Sterile, without Rack
PJ27S	PhycoLink® Streptavidin-Allophycocyanin Conjugate
PJRS25	PhycoLink® Streptavidin-R-Phycoerythrin
DBLYS0B	Human BAFF/BLyS/TNFSF13B
DCX130	Recombinant Human CXCL13/BCA Quantikine ELISA
0855371	Complement Anti C3 Polyclonal Antibody, Anti-Guinea Pig, Goat
557831	APC-CY7 Mouse Ant-Human CD14
A9418-500G	Albumin from Bovine Serum (BSA), Bio Reagent, suitable for cell culture, lyophilized powder, >96% (agarose gel electrophoresis)
F8775	FluoSpheres© NeutrAvidinU labelled microspheres, 1.0 cm, red fluorescent (580/605), 1% solids
F8776	FluoSpheres© NeutrAvidin Labelled Microspheres, 1.0 um, yellow-green fluorescent (505/515) 1% solids
CL4051	Low-Tox® Guinea Pig Complement, Lyophilized
0855371	Complement Anti C3 Polyclonal Antibody, Anti-Guinea Pig, Goat
00-4333-57	1X RBC Lysis Buffer
557831	APC-Cy™7 Mouse Anti-Human CD14
305112	Pacific Blue™ anti-human CD66b Antibody
557943	Alexa Fluor® 700 Mouse Anti-Human CD3

15065	RosetteSep™ Human NK Cell Enrichment Cocktail
H8889-500ML	Histopaque®-1077 Hybri-Max® liquid, Sterile-Filtered, Hybridoma Tested
86450	SepMate™-50 (IVD) 50 mL Centrifuge Tube for density gradient centrifugation
B7651-5MG	Brefeldin A from Penicillium Brefeldianum, >99%
554724	GolgiStop®
GAS001S100	Fixation Medium (Medium A)
GAS002S100	Permeabilization Medium - General, (MEDIUM B)
555802	Cy-Chrome U labelled anti-human CD107α (LAMP-1)
557747	PE-Cy™7 Mouse Anti-Human CD56
554702	APC Mouse Anti-Human IFN-gamma
31985070	Opti-Mem
P7539	Penicillin streptomycin solution
H0887	HEPES solution
1114544	Lymphoprep
109-055-043	AP conjugated affinipure goat anti-human IgM
109-055-098	AP conjugated affinire pure goat anti-human IgG, Fc
31350010	Beta-mercapto ethanol
P9187	OPD peroxidase substrate
R0883	RPMI-1640 medium
15575020	0.5M EDTA
25030081	L-Glutamine

E2691	Fugene transfection reagent
16176	Luciferase kit
ATCC T1B-202	THP-1 cells
B94987	Vi-CELL reagent
550078	PE Mouse Anti-Human MIP-1ß
90283	Qsol buffer